Is fouling a determinant of invasion success? - A comparison of the susceptibility to fouling between native and non-native populations of *Gracilaria vermiculophylla*

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Abstract

The number of introduced species, also called non-native or invasive species, has substantially increased in both terrestrial and aquatic ecosystems worldwide in past years. One possible reason for invasion success, i.e. the permanent establishment and spread after introduction, could be a high resistance towards biotic stressors such as herbivory. In the marine environment epibiosis (the colonization of living surfaces or exoskeletons by sessile organisms) is a common stressor and a high resistance towards fouling could explain invasion success in introduced species.

For the present doctoral thesis, I used common garden experiments to compare the susceptibility to fouling between native and non-native populations of the red macroalga *Gracilaria vermiculophylla* (ohmi) Papenfuss. *Gracilaria vermiculophylla* is native in the Northwest Pacific and occurs as a non-native species in other areas on the Northern hemisphere. For the comparative experiments, I used four native *G. vermiculophylla* populations (from the Yellow Sea and the Northwest Pacific) and five non-native populations (four from Europe: North Sea, Baltic Sea and the British Channel and one from North America: Northeast Pacific). To compare the susceptibility to fouling between these different populations, both laboratory and field experiments were performed. In laboratory experiments, settlement rates by diatoms and *Ceramium* filaments, both of which came from both distributional ranges of *G. vermiculophylla*, were quantified on substrata covered with *G. vermiculophylla* surface extracts as well as on living *G. vermiculophylla* individuals from all populations in different seasons. In field experiments, *G. vermiculophylla* individuals from all populations were individually enclosed in dialysis membrane tubes and exposed to the natural fouling at both sites in the native and non-native distributional range to assess the abundance and composition of foulers establishing on the tubes. In parallel to the field experiments, a monitoring was conducted in both distributional ranges (native and non-native) to quantify and characterize the fouling communities that are associated with *G. vermiculophylla*. Additionally, I investigated seasonal variations in antifouling defences against diatoms and against *Ceramium* in *G. vermiculophylla* from the Baltic Sea. This was done by quantifying fouling rates of these two species on *Gracilaria* surface extracts in different months of the same year as well as by correlating the strength of antifouling defences with the prevailing fouling pressure in the field.

My study revealed that fouling pressure was significantly higher at a site in the non-native than at a site in the native range. Florideophyceae, e.g. *Ceramium* and *Polysiphonia*, as well as Ciliata were the most common taxonomic groups in the natural fouling consortia that are associated with *G. vermiculophylla* in its native distributional range, while Ciliata and

Gymnolaemata were most common on *G. vermiculophylla* in its non-native range. Furthermore, I demonstrated that living thalli and surface extracts from non-native populations of *G. vermiculophylla* were better defended against the foulers tested (diatoms and *Ceramium* filaments) - regardless of their origin - than those from native conspecifics. Moreover, fouling on the outside of the membrane tubes that were filled with *Gracilaria* individuals was obviously influenced by compounds, which were released by the alga and diffused through the membrane. At both study sites (in the native and non-native range) dialysis membrane tubes containing non-native *G. vermiculophylla* were less fouled than those containing native conspecifics. Additionally, the fouling resistance of *G. vermiculophylla* against diatoms and *Ceramium* filaments differed clearly with season. I could show that fluctuations in fouling resistance mediated by dichloromethane (DCM) extracts against *Ceramium,* correlated with fluctuations in the intensity of epiphytism by this fouler on *G. vermiculophylla*. Finally, diatoms were generally deterred by *Gracilaria* surface extracts made with hexane, while *Ceramium* filaments were generally deterred by extracts gained with both hexane and DCM.

This the first study that compares the susceptibility to eukaryote microfouling, macrofouling and natural fouling between native and non-native populations of an aquatic species. Further, this work gives the first example that non-native individuals of an aquatic species are better defended against fouling than native conspecifics and suggests that an enhanced defence against fouling after introduction could explain – at least in parts - the invasion success of *G. vermiculophylla*. Additionally, this work demonstrates that the chemical antifouling defence in *G. vermiculophylla* varies with season and is based on multiple compounds that have different polarities. Finally, the new technique of enclosing macroalgae in dialysis tubes represents a simple, efficient and accurate way to test for the presence of chemical antifouling defences in these organisms and could possibly be applied to other algal species. The study thus provides new insights into the invasion ecology of macroalgae.

Zusammenfassung

Weltweit hat die Anzahl von neu eingeführten Arten, auch als nicht-native oder invasive Arten bezeichnet, die sich in terrestrischen und in aquatischen Ökosystemen etablieren und ausbreiten konnten, in den letzten Jahren deutlich zugenommen. Ein möglicher Grund für den Invasionserfolg vieler Arten könnte deren hohe Resistenz gegenüber biotischen Stressoren, wie zum Beispiel Herbivorie, sein. Im Meer ist Epibiosis (auch etwas ungenauer als Aufwuchs oder Fouling bezeichnet), d.h. die Besiedlung lebender Oberflächen bzw. von Exoskeletten lebender Tiere durch sessile Organismen, ein häufiger Stressor und eine hohe Resistenz gegenüber Aufwuchs könnte den Invasionserfolg von eingeführten Arten zumindest in Teilen erklären.

Für die vorliegende Doktorarbeit habe ich eine vergleichende Studie mittels eines „common garden“ Ansatzes durchgeführt, um damit die Anfälligkeit gegenüber Epibiosis zwischen Individuen aus nativen und nicht-nativen Populationen der roten Makroalge *Gracilaria vermiculophylla* (ohmi) Papenfuss zu vergleichen. *Gracilaria vermiculophylla* kommt nativ im Nordwest-Pazifik vor und hat sich als invasive Art in weiteren Meeresgebieten auf der Nordhalbkugel ausgebreitet. Für das Vergleichsexperiment habe ich vier native *G. vermiculophylla*-Populationen (aus dem Gelben Meer und dem Nordwest-Pazifik) und fünf nicht-native Populationen (aus Europa: Nordsee, Ostsee und Ärmelkanal sowie eine Population aus Nordamerika: Nordost-Pazifik) beprobt. Um die Anfälligkeit dieser verschiedenen Populationen gegenüber Epibiosis zu vergleichen, wurden sowohl Labor- als auch Feldexperimente durchgeführt. In den Laborexperimenten wurde die Anzahl der Siedlungsereignisse von Diatomeen und *Ceramium*-Filamenten, beide sowohl aus dem nativen als auch aus dem nicht-nativen Verbreitungsgebiet von *G. vermiculophylla,* ermittelt. Dies geschah auf Oberflächen, die mit Extrakten aus *G. vermiculophylla* beschichtet wurden, sowie auf lebenden *G. vermiculophylla*-Individuen aus allen beprobten Populationen. Die Experimente wurden zusätzlich in verschiedenen Jahreszeiten wiederholt. In den Feldexperimenten wurden lebende Individuen von *G. vermiculophylla*, ebenfalls aus allen beprobten Populationen, einzeln in Dialysemembranschläuche eingeschlossen und an Standorten im nativen und nicht-nativen Verbreitungsgebiet der Art der natürlichen Besiedlung durch Epibionten ausgesetzt. Parallel zu den Feldexperimenten wurde an beiden Versuchsstandorten ein Monitoring durchgeführt, um die Abundanz und Zusammensetzung der mit *G. vermiculophylla* assoziierten Aufwuchsgemeinschaften zu erfassen. Darüber hinaus habe ich die Saisonalität in der Abwehr von Diatomeen und *Ceramium* durch *G. vermiculophylla* aus der Westlichen Ostsee untersucht und eine Korrelation zwischen der Stärke der Abwehr und dem vorherrschenden Besiedlungsdruck vorgenommen.

Meine Studie hat gezeigt, dass der Besiedlungsdruck an den Standorten im nicht-nativen Verbreitungsgebiet signifikant höher war als an den Standorten im nativen Verbreitungsgebiet. Florideophyceae, z. B. *Ceramium* und *Polysiphonia*, sowie Ciliaten waren die häufigsten Aufwuchsorganismen im nativen Verbreitungsgebiet, während im nicht-nativen Verbreitungsgebiet Ciliata and Gymnolaemata am häufigsten auf *G. vermiculophylla* zu finden waren. Weiter habe ich gezeigt, dass lebende Thalli aus nicht-nativen Populationen bzw. Oberflächen, die mit Extrakten aus nicht-nativen *G. vermiculophylla*-Individuen beschichtet wurden, besser gegen die getesteten Aufwuchsorganismen (Diatomeen und *Ceramium*-Filamente) verteidigt waren als solche aus dem nativen Verbreitungsgebiet. Dies war der Fall unabhängig davon woher die Aufwuchsorganismen stammten. Darüber hinaus stellte sich heraus, dass die Besiedlung der Membranschläuche, die mit Gracilaria-Individuen gefüllt wurden, durch Stoffe beeinflusst wurden, welche von innen durch die Membran diffundierten. An beiden Untersuchungsstandorten (im nativen und nicht-nativen Verbreitungsgebiet) waren die Dialysemembranschläuche, die nicht-native *G. vermiculophylla* enthielten, weniger besiedelt als die, die Individuen aus dem nativen Verbreitungsgebiet enthielten. Außerdem variierte die Resistenz von *G. vermiculophylla* gegenüber Bewuchs durch Diatomeen und *Ceramium*-Filamente deutlich mit den Jahreszeiten. Allerdings korrelierten nur die Schwankungen in der Aktivität von Dichloromethan (DCM)-Extrakten gegen *Ceramium*-Filamente mit den gemessenen Fluktuationen im Besiedlungsdruck durch diese Alge. *Gracilaria*-Oberflächenextrakte, die mit Hexan hergestellt wurden, hatten einen negativen Effekt auf Diatomeen, während die Anheftung von *Ceramium*-Filamente durch Hexan und DCM-Extrakte reduziert wurde.

Dies ist die erste Studie, die die Resistenz gegenüber eukaryotischem Mikroaufwuchs und gegenüber Makroaufwuchs unter Labor- und Feldbedingungen zwischen nativen und nicht-nativen Populationen einer aquatischen Art verglichen hat. Weiter gibt diese Arbeit das erste Beispiel dafür, dass nicht-native Individuen einer aquatischen Art besser gegen Aufwuchs verteidigt sind als ihre nativen Artgenossen. Dies legt die Vermutung nahe, dass eine gesteigerte Abwehr gegen Aufwuchs den Invasionserfolg von *G. vermiculophylla* zumindest in Teilen erklären könnte. Zusätzlich zeigt diese Arbeit, dass die Stärke der chemischen Abwehr bei *G. vermiculophylla* mit den Jahreszeiten variiert und das verschiedene Verbindungen, mit unterschiedlichen Polaritäten, involviert sind. Der neue Ansatz Makroalgen in Dialyseschläuche einzuschließen, um sie der natürlichen Besiedlung an einem Standort auszusetzen, stellt einen einfachen, effizienten und verlässlichen Weg dar, um deren chemische Abwehr zu untersuchen und kann sicherlich auch auf andere Arten angewandt werden.

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1. General Introduction

An introduced species is defined as a species that has been introduced into geographic regions beyond its native range directly or indirectly through human activities. By this it overcame major geographical barriers, such as mountains, rivers and artificial canals, and has become successfully established in regions where it previously has been absent from (Boudouresque and Verlaque 2002, Williamson and Fitter 1996). An introduced species is considered invasive when it incurs or is likely to incur ecological and/or economic negative impacts (Williams and Smith 2007). However, it is difficult to predict/assess this, because most often studies are lacking that document the impacts of an introduced species in its new range. Identifying a species as invasive is therefore most often a subjective interpretation rather than an objective finding (Hammann 2014).

In this doctoral thesis, I will therefore use the term ‘non-native’ as a description for species that have been introduced to a new habitat through human activities and that also have established and spread in the new range, and will use the term ‘invasion’ as a description for this event. However, they do not necessarily mean that they so far also had negative consequences for the ecosystems to which they were introduced.

The amount of successful marine biological invasions worldwide increases exponentially, and approximately 20% of which are represented by macroalgae (Schaffelke et al. 2006). However, many marine non-native species cause severe ecological or economic impacts. Numerous studies have revealed that non-native species can have negative ecological consequences at both the species and the community levels. At the species level, for example, non-native species compete with native species for limited resources (e.g. light, nutrients and substrate), leading to reduced growth or reproduction of the native species (Thomsen et al. 2009). At the community level, non-native species can lead to changes in species richness, evenness and diversity (Crooks and Khim 1999). Economically, negative impacts can occur on local aquaculture, fisheries, marine infrastructure and tourism. For example, the introduced filamentous macroalgae foul on water intakes and underwater constructions, drifting macroalgal mats clog or even hamper fishing operations and decrease the recreational value of beaches and increase costs for cleanings (Freshwater et al. 2006, Sakai et al. 2001). To manage biological invasions and to alleviate their negative effects, the necessity to understand the mechanisms determining success or failure of such anthropogenic introductions is a pressing task in invasion ecology (Hewitt et al. 2007, Schaffelke et al. 2006).

1.1 The invasion process

Introductions of species into new environments have been occurring for a long time and appear to occur with increased frequency in coastal regions of the world due to the fast and far-ranging transportation possibilities at the turn of the 20th to the 21st century (Carlton and Geller 1993). During the last two decades, there has been a surge of literature studying the success of introduced species and the patterns and processes behind biological invasions (Carlton 1996, Grosholz 2002, Mallon et al. 2015).

Carlton (1996) suggested six interrelated processes (changes in donor regions, emerging of new donor regions, changes in recipient region, occurrence of invasion windows, stochastic inoculation events, and dispersal vector changes) to understand the mechanisms behind biological invasions. The first process means that the donor region may change environmentally, which could lead to the fact that more populations increase in pre-existing species, such that more individuals would be available to interface with a transport mechanism (such as ballast water) or that local species expand into previously uninhabitable donor areas where they are then available for transport. Alternatively, the environment may not change, but a new species may become available for transport for the first time. This is, in essence, a 'hub and spoke' model, where a species enters a high-intensity transport hub potentially to be carried along a corridor to another transport hub, which lies at the end of the spoke - which in turn is another hub, and from which radiate out new corridors. The second process means that new donor regions become available due to new species with different adaptive capacities than previously-transported ones or new populations of already introduced species, but from other donor regions become available for transport. The third process means that any environmental change, such as a decline in water quality due to pollution in a recipient region can lead to altered ecological, biological, chemical, or physical states, and thus change the region’s susceptibility to invasions. Then, invasions occur when the proper combination of physical, chemical, biological, or ecological conditions occur to allow colonizing and permit the long-term establishment of reproducing populations. The fifth mechanism is the release of a large number of inoculants (adults, larvae, spores) into the recipient region what increases the potential reproductive success of the introduced species.

Finally, an increase in the size, the speed, and the quality of a vector, such as a ship, can lead to increased inoculant species diversity, increased inoculant species abundance or increased number of healthy and reproductive post-transport individuals.

Heger (2001) built a model of stages with which the invasion process can be visualized as a staircase. A species must overcome several steps (arrival in the new area, spontaneous establishment, permanent establishment and spreading in the new area) to advance from one stage to the next. Nyberg (2007) modified this model and suggested that the invasion process can be divided into three stages, i.e., introduction, establishment and invasion (Figure 1). The success of introduction largely depends on human activities, i.e. the species needs to be transported by a vector to a new area (Munro ALS 1999). To proceed from introduction to establishment, at least one individual of the introduced population must succeed to grow and reproduce independently in the new area. A species is regarded as established in the new area when they have developed a self-sustaining population (Boudouresque and Verlaque 2002). Once established, the introduced species may spread naturally (e.g. by currents) or by human activities (Sakai et al. 2001). As said earlier, if the introduced species becomes abundant in the recipient region and has negative impacts on the environment and/or economy it is referred to as invasive (Nyberg and Wallentinus 2005).

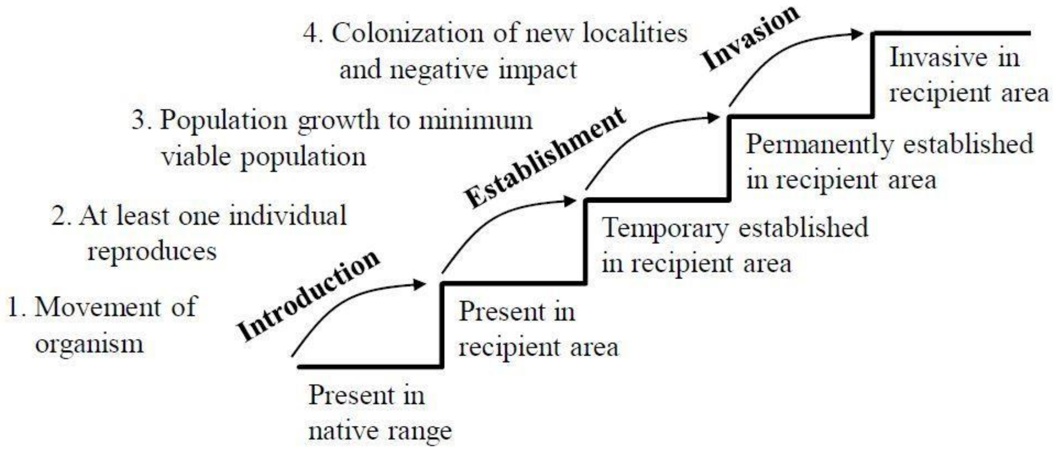

Figure 1. The different stages of the invasion process and the requirements to go from one stage to the next (Nyberg 2007).

Williamson and Fitter (1996) suggested a statistical regularity to predict the proportion of transported species that reach the different levels of the invasion process, i.e. ‘tens rule’, which states that, on average, 1 out of 10 of those that are transported become introduced, 1 out of 10 those introduced become established, and that 1 out of 10 those established become invasive. Although it is generally agreed that as yet we can hardly ever predict the success of introduced individuals, there is no doubt that there are statistical regularities to invasions, while the suggested factors of the ‘tens rule’ has been critically discussed.

1.2 Why do species become non-native?

To manage biological invasions and to alleviate their negative effects, it is of high importance to understand the mechanisms that determine the success or failure of invasions: Why do certain species establish in new habitats successfully while others fail (Johnson and Chapman 2007)? Which factors facilitate the establishment and spread of introduced species in a new habitat (Hu and Juan 2014, Nyberg and Wallentinus 2009)?

In the marine environment, a large number of studies have revealed that the success of introduced species depends on a number of factors. For instance, most species establish in new suitable areas with similar climate and other abiotic conditions as their native regions (Wikström 2004); some species have a versatile reproductive strategy, a broad ecological tolerance and an enhanced resistance towards enemies (e.g. competitors, predators, fouling organisms and pathogens) what allows them to withstand adverse conditions in new regions with, e.g., climate and salinity regimes different from their native regions (Hu and Juan 2014).

Hitherto, there is a variety of hypotheses that has been discussed to elucidate invasion mechanisms in terrestrial environments (Hierro et al. 2005, Joshi and Vrieling 2005), such as the enemy release hypothesis (ERH) (Keane and Crawley 2002), the evolutionarily increased competitive ability (EICA) hypothesis (Blossey and Nötzold 1995) and the novel weapons hypothesis (Callaway and Ridenour 2004). Some of these hypotheses have been tested and were supported also for the case of invasions by marine macroalgae.

1.2.1 Resistance towards biotic and abiotic stressors

Several studies have demonstrated that biotic stressors, such as grazing and fouling, can be determinant for macroalgal invasions (Hammann et al. 2013, Saha et al. 2016). Of these two stressors, grazing has been studied much more frequently. Originally, all these studies have been done to compare the susceptibility of non-native and co-occurring native species only in the introduced range. For example, Weinberger et al. (2008) found that in the Kiel Fjord two local invertebrate grazers (*Idotea baltica* and *Littorina littorea*) clearly prefer *Fucus vesiculosus*, when it is present, over the non-native red macroalga *Gracilaria vermiculophylla*. Enge et al. (2012) reported that native generalist herbivores (e.g. *Idotea granulosa* and *Gammarus locusta*) from the Swedish west coast preferred native algae (e.g. *Lomentaria clavellosa* and *Ceramium virgatum*) over the non-native red macroalga *Bonnemaisonia hamifera* in pairwise feeding experiments. During the past decade, studying non-native species at a biogeographic scale in both their native and non-native ranges have been considered very crucial for understanding exotic species invasions and studies that investigated the relevance of herbivory for the invasion success of non-native species therefore have focused on comparisons of the effects of herbivory in both the native and the non-native range. For example, Wikström et al. (2006) revealed that the brown macroalga *Fucus evanescens* was a preferred host in its native range, and it was less preferred in its non-native range in laboratory choice experiments with generalist grazers - the isopod *I. granulosa* and the littorinid gastropod *Littorina obtusata* - than co-occurring native macroalgae. In addition, Hammann et al. (2013) showed that in no-choice feeding assays herbivorous snails from both, the native range in Asia (*Littorina brevicula*) and from the non-native range in Europe (*L. littorea*), consumed less non-native *G. vermiculophylla* populations than their native conspecifics.

Similar to herbivores, fouling organisms can also have the potential to determine the success or failure of macroalgal invasion. However, interactions between non-native macroalgae and resident fouling organisms have been very rarely studied. Comparison study by Strong et al. (2009) showed that in Strangford Lough, Northern Ireland, thalli of the non-native brown macroalga *Sargassum muticum* were as heavily overgrown by seasonal blooms of ectocarpoid fouling as native macroalgal species. This suggests that non-natives are not always released from fouling. The observations made by Saha et al. (2016), who focused on seaweed-bacteria interactions, showed that non-native individuals of *G. vermiculophylla* are well defended against co-occurring bacteria from the invaded range but, at the same time, have a weakened capacity to defend against bacteria from the home range. The authors suggested that confrontation with new enemies during the invasion process may trigger a rapid defence adaptation in introduced seaweeds, which, in turn, could be a pre-requisite for invasion.

The invasion success of macroalgae is also based on various physiological traits that mediate resistance against abiotic stress (Johnson and Chapman 2007), such as the ability to tolerate darkness, extreme temperatures, salinity fluctuations (Raikar et al. 2001), starvation (Nyberg and Wallentinus 2009), ultraviolet radiation (Roleda et al. 2012) and grazers (Weinberger et al. 2008). Wikström et al. (2002) found that in Swedish waters the non-native *F. evanescens* can survive and grow in a wide salinity range from 10 to 24 psu, while optimal salinities for marine organisms are between 30 and 35 psu. Hu and Juan (2014) reported that *G. vermiculophylla* can still survive at extreme temperatures such as 2 and 35 ℃, while the optimum temperature range for its growth is from 11 to 30 ℃. In addition, they also reported that *G. vermiculophylla* is well adapted to low light conditions, which allow this seaweed to survive during transportation in dark condition, e.g. in ballast water.

1.2.2 Mechanisms that facilitate invasion success

The question remains why some species can invade successfully and which exact factors allow the spread and establishment of such non-native species. So far, various hypotheses have been put forward to address this question.

The Enemy Release hypothesis (ERH) states that when species are introduced into new habitats they are confronted with an abiotic and biotic environment that can be substantially different from the one they adapted to over evolutionary time scales. This may include the absence of antagonists, with which they co-evolved at their site of origin, while resident predators or parasites cannot use the newly introduced resource. As a consequence, non-natives should have an advantage over indigenous species (Keane and Crawley 2002).

The Evolution of Increased Competitive Ability (EICA) hypothesis which is an expansion of ERH argues that non-native species which are released from their native enemies do not need to defend or, at least, do not need to invest as much energy into warding-off predators and parasites as in their home range. This would allow them to invest more energy into growth, reproduction or into the compensation of the effects of environmental stress. This should make them stronger competitors than the native species (Blossey and Nötzold 1995).

Along another lane of thought, the Novel Weapons hypothesis by Callaway and Ridenour (2004) suggests that non-native species should be less sensitive to enemies in the invaded habitat, because they possess new or stronger biochemical defence mechanisms than the species which are already living at the new site.

These hypotheses have already been examined in terrestrial systems with organisms such as spermatophytes and their feeding antagonists and competitors (Keane and Crawley 2002), but very little research has so far been conducted with marine species. More than this, as mentioned above, most studies on the interactions between non-native species and their enemies in terrestrial and aquatic systems considered only herbivores (Cappuccino and Carpenter 2005, Wikström et al. 2006), and less is known about resistance against fouling organisms, which only occur in aquatic systems. All hypotheses described above should also hold true for defences against fouling organisms and they are worth investigating, since putative differences in the defences against fouling organisms between non-native and native individuals and/or populations of the same species have so far been widely ignored.

1.3 Marine epibiosis and its consequences for host macroalgae

In the marine environment, in particular in benthic habitats which are located within the euphotic zone, competition for light, space, nutrients and other resources is high (Wahl 1989) and sessile and even mobile organisms ('basibionts') are prone to colonization by other sessile life forms ('epibionts', 'fouling organisms' or 'foulers'). The latter comprise many different taxa from both the animal (epizoan) and the plant (epiphyte) kingdom, such as invertebrates, epiphytic macroalgae, microalgae, protists and bacteria. This ubiquitous and facultative phenomenon of an association between epibionts and basibionts is known as epibiosis (Wahl 1989).

In some cases, associations between epibionts and basibionts can be beneficial to both of them (Egan et al. 2001). For instance, some epibiotic bacteria are known to mediate further colonization processes by microfoulers which contribute to the host alga's protection system against macrofouling (Lachnit et al. 2009). Dobretsov and Qian (2002) isolated a *Vibrio* sp. strain from green macroalga *Ulva reticulata* that significantly inhibits the settlement and metamorphosis of polychaete larvae. Additionally, Nasrolahi et al. (2012) revealed that surface associated monospecies bacterial biofilms as well as natural microbial assemblages from the macroalga *F. vesiculosus* repelled barnacle larvae. However, in other cases, associations between epibionts and basibionts have been found to be disadvantageous for the host (Wahl 2008). For example, some bacteria are potential pathogens. Vairappan et al. (2001) showed that *Alteromonas* sp. isolated from the Japanese kelp *Laminaria religiosa* can cause the bleaching disease in this kelp species during spring. Furthermore, some epibionts could mechanically damage host tissues by anchoring and thallus penetration. For example, Gonzalez and Goff (1989) found that the red epiphytic alga *Microcladia coulteri* penetrates the surface tissues of the brown alga *Egregia menziesii*. In addition, it has been reported that high densities of epibionts are harmful to macrophytic hosts because they reduce diffusion rates of oxygen, carbon dioxide and nutrients to the thallus and decrease the amount of light available for photosynthesis. They physically inhibit sporulation, decrease thallus flexibility, and increase the palatability of the thalli to herbivores (da Gama et al. 2008, Pereira et al. 2003). For example, the epiphyte *Polysiphonia lanosa* reduced reproduction success of its host red macroalga *Ascophyllum nodosum* due to the physical blockage of receptacles (Kraberg and Norton 2007). Furthermore, epibiosis by the bryozoan *Membranipora membranacea* and by haplosclerid sponges increased the susceptibility of the red macroalga *Cryptonemia seminervis* to consumption by sea urchins and amphipods. This could be due to an increased nutritional value of fouled compared to clean algal tissue (da Gama et al. 2014).

1.4 Antifouling mechanisms in macroalgae

Since fouling organisms can have strong negative effects on macroalgal hosts, the latter should be selected for efficient physical or chemical defence that minimize colonization of their body surfaces by fouling organisms that confer these negative effects (da Gama et al. 2014).

Periodical epithallus sloughing, the best known physical defence against epibionts, has been reported in numerous species of macroalgae, such as the brown alga *Sargassum* spp. (Yamamoto et al. 2013), the red alga *Dilsea carnosa* (Nylund and Pavia 2005), as well as the green alga *Ulva intestinalis* (McArthur and Moss 1977). Additionally, the covering of surfaces with gelatinous mucus that reduces adhesion strength is another common physical antifouling mechanism among brown, red and green macroalgae (Chapman et al. 2014).

It has been shown that green, brown and red macroalgae can chemically defend against surface colonization by the formation and emission of reactive oxygen species (ROS), commonly referred to as ‘oxidative burst’ (da Gama et al. 2014). For example, certain lipopolysaccharides (LPS) released by *Salmonella arbotus equi* can induce an oxidative burst in the brown macroalga *Laminaria digitata* which contributes to the host alga’s protection against epibacteria (Küpper et al. 2006).

In addition to this, metabolites have been isolated from macroalgae that deterred the settlement of fouling organisms, but most studies of this kind have focused on substances gained from whole-cell extracts (Amade and Lemée 1998). However, metabolites which are produced to reduce fouling must either be present on the surface of the algal host or must be released into the surrounding water at ecologically relevant concentrations (Nylund et al. 2007). As a consequence, some studies focused only on antifouling activities in surface-associated metabolites (Dworjanyn et al. 2006). For example, surface extracts of the red alga *Delisea pulchra* and the green alga *Caulerpa filiformis* inhibit the settlement of *Polysiphonia* sp. spores (Nylund et al. 2007). Studies with the brown alga *F. vesiculosus* identified polar and non-polar compounds gained from the alga's surface that have the potential to control epibiotic biofilms (Saha et al. 2011, 2012). Rickert et al. (2015) showed that surface-extracted metabolites from the brown algae *F. vesiculosus* and *Fucus serratus* deter the larval settlements of the barnacle *Amphibalanus improvisus* and the bivalve *Mytilus edulis*.

1.5 Seasonality in macroalgal antifouling defences

Seasonal fluctuations in the concentrations of bioactive antifouling metabolites (Amade and Lemée 1998) and in the antifouling defence strength in macroalgae have already been documented in several species (Hellio et al. 2004, Maréchal et al. 2004, Rickert et al. 2015). For instance, concentrations of caulerpenyne, which is the major antifouling secondary metabolite in the green alga *Caulerpa taxifolia*, is regulated by light intensity and water temperature (Amade and Lemée 1998). Further, previous studies on *F. vesiculosus* revealed that anti-settlement activities of surface extracts against microfouling vary seasonally (Saha and Wahl 2013, Wahl et al. 2010). If the production of active chemical compounds consumes energy that is then not available for other life processes (Dworjanyn et al. 2006), defence strength should vary with seasonal shifts in abiotic factors such as light intensity and water temperature (Lehvo et al. 2001) and with shifts in biotic factors such as grazing and fouling pressure (Rickert et al. 2015, Schauer et al. 2003). In temperate marine environments, fouling pressure and also the composition of the establishing fouling communities can vary seasonally (Wahl et al. 2010). It has been repeatedly suggested that the actual fouling pressure acts as a driving force in regulating fouling resistance (Hellio et al. 2004, Maréchal et al. 2004, Rickert et al. 2016). However, only few studies have directly related algal antifouling defences to the *in situ* fouling pressure. For example, Maréchal et al. (2004) showed that the antifouling activity of whole-cell extracts gained from the brown alga

Bifurcaria bifurcata against cypris larvae of the barnacle *Balanus amphitrite* fluctuated with season and reached a peak in in summer corresponding to the maximum in fouling pressure. Furthermore, Rickert et al. (2016) showed that in *F. vesiculosus* and *F. serratus* the activity of surface extracts against microfouling varied with season and was tend to be in phase with fluctuations in the fouling pressure exerted by microfouling.

1.6 Study species: the non-native macroalga *Gracilaria vermiculophylla*

The perennial red macroalga *Gracilaria vermiculophylla* (ohmi) Papenfuss is an important agar producing economic seaweed. In some countries, it is cultivated in industrial mariculture for agar production and can is also consumed by humans (Sánchez-Machado et al. 2004, Villanueva et al. 2010). This seaweed originates from the Northwest Pacific (Tseng and Xia 1999). During the last three to four decades it has invaded coastal areas in a) the eastern Pacific, such as California, Mexico and British Columbia, Canada (Bellorin et al. 2004, Saunders 2009), b) the western Atlantic, such as the coasts of the US states Virginia, North Carolina and New England (Freshwater et al. 2006, Thomsen et al. 2006) and c) the eastern Atlantic, such as France, Spain, Portugal, Germany, and Sweden (Guillemin et al. 2008, Weinberger et al. 2008), and can now be found in a considerable proportion of coastal habitats on the northern hemisphere. This species can grow in different kinds of habitats, including the intertidal zone, the upper subtidal, soft bottom sediments and hard substrata (small stones, shells, mussels) (Nyberg 2007). It is irregularly branched, with three to four

Figure 2. The perennial red macroalga *Gracilaria vermiculophylla* in Kiel, Germany (non-native range) (left), and in Qingdao, China (native range) (right).

orders of branches and can reach a size from 10 cm to 1 m in length. Its colour varies from purplish brown to dark brown and sometimes to greenish or yellowish (Tseng and Xia 1999) (Figure 2).

Gracilaria vermiculophylla has been placed among the four most potent invaders out of 114 non-native macroalgal species that occur in Europe (Nyberg 2007). This ranking goes back to the assessment of 13 species traits that presumably serve to facilitate dispersal and establishment of introduced species as well as to aggravate their ecological impacts (Nyberg and Wallentinus 2005).

During the last years, *G. vermiculophylla* has proven to be a particularly suitable marine model organism for tests of ecological theories that are related to invasion ecology. For example, it has been demonstrated that the invasion process of this species was associated with a severe loss in genetic diversity in the non-native populations (Kim et al. 2010), which, however, proved to be more resistant towards herbivory (Hammann et al. 2013, 2016) and heat stress (Hammann 2014).

Other empirical studies revealed that further species traits, such as a versatile reproductive strategy and the ability to grow also detached from a substratum, are important factors that promote successful invasions in *G. vermiculophylla*. For instance, this alga has an isomorphic life cycle and can reproduce sexually and asexually (vegetative fragmentation). It shows a rapid growth from germling (a visible cystocarp formation after 18 days and carpospores release after 35 days) to sexual maturity (dioecious gametophyte mature after three to four months) (Hu and Juan 2014, Rueness 2005). In natural habitats, a fragmentation of this alga even as small as 1 mm can survive and reproduce after detachment.

1.7 Thesis outline

The aim of the present thesis is to investigate whether individuals from native and non-native populations of a marine plant species differ in their susceptibility to fouling. In my study, I used the macroalga *G. vermiculophylla* as a model organism. Since seaweeds are exposed to fouling by invertebrates, epiphytic macroalgae, microalgae, protists and bacteria and since differences in the colonizer pools between the sea areas studied in here are well documented for invertebrates, macroalgae and microalgae, but not for protists and bacteria, I focused on the first three groups of fouling organisms.

In common garden experiments, I examined whether fouling rates and the compositions of the establishing fouling communities on the algal thalli generally differ

a) between individuals from native and non-native populations of *G. vermiculophylla*,

b) between the native and non-native distributional range of *G. vermiculophylla* and

c) between different seasons.

Therefore, both laboratory and field fouling experiments were performed.

Algal material from five non-native and four native populations was collected for this study. The five non-native populations were located in Northern Germany (Kiel and Nordstrand), Western France (Belon and Pouldouran) and in Western Canada (Port Moody), while the four native populations were located in Eastern China (Qingdao and Rongcheng in the Shandong region) and Japan (Akkeshi and Tokyo).

All experiments were carried out in the native range (Rongcheng/China or Akkeshi/Japan) as well as in the non-native range (Kiel/Germany) of this alga.

Both living thalli and surface extracts from *G. vermiculophylla* were tested in laboratory experiments, which compared the frequency of attachment events of epiphytic diatoms and *Ceramium* filaments on individuals from native and non-native populations of *G. vermiculophylla.* These assays were conducted in a) Kiel, Germany and b) Rongcheng, China in different seasons. Both types of micro- and macrofoulers were collected in Kiel and in Rongcheng (**Paper I**).

In parallel, living thalli from all *G. vemiculophylla* populations, which were enclosed in tubes made of dialysis membrane, were exposed for two to three weeks to natural fouling in a) Kiel Fjord, Germany and b) Akkeshi Bay, Japan. The new technique of enclosing *G. vermiculophylla* in dialysis membrane tubes was applied to prevent *Gracilaria* spores or alga-associated microorganisms from entering the water column and to evaluate the relevance of chemical compounds for the antifouling defence system of *G. vermiculophylla*. In addition to these field fouling experiments, living algal thalli and inert surfaces were repeatedly submerged at sites in both distributional ranges of *G. vermiculophylla* to quantify regional fouling pressure during the experiments (**Paper II**).

In the laboratory experiments conducted in this study, antifouling defences in *G. vemiculophylla* individuals from all tested populations, regardless of their origin, were observed to differ in strength between summer and autumn. Thus, a further study was conducted to test whether the seasonal fluctuations in the antifouling activity of this alga correlate with the prevailing fouling pressure in the field. For this, surface extracts from monthly collected *G. vermiculophylla* in the Kiel Fjord were used in laboratory settlement assays with the diatom *Stauroneis constricta* and the red alga *Ceramium tenuicorne,* which both stem from the same site. During the same time period, living thalli and inert surfaces were submerged in the Kiel Fjord once a month to record natural fouling pressure (**Paper III**).

All these were done to answer the following questions:

1. What species can be found in the natural fouling consortia that establish on *G. vermiculophylla* in its native and its non-native range? Do these assemblages generally differ in diversity and biomass? (**Paper II**)

2. Do native and non-native populations of *G. vermiculophylla* differ with respect to their susceptibility to fouling? (**Paper I** and **II)**

3. Does the strength of antifouling defences in *G. vermiculophylla* vary with season and do seasonal fluctuations in the strength of antifouling defences correlate with fouling pressure? (**Paper I** and **III)**

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2. Publications and Contributions of Authors

Parts of this doctoral thesis have been published or submitted:

Paper I

Wang S, Wang G, Weinberger F, Bian D, Nakaoka M, Lenz M (**2016**) Anti-epiphyte defences in the red seaweed *Gracilaria vermiculophylla*: non-native algae are better defended than their native conspecifics. **Journal of Ecology doi: 10.1111/1365-2745.12694**

ML, FW and SW conceived and designed the experiments. SW performed the experiments and collected algal material. GW, DB and MN contributed to algal collection and the labs in China and Japan for bioassays. ML and SW performed statistical analyses. SW wrote the manuscript, and ML and FW contributed to revisions.

Paper II

Wang S, Weinberger F, Bian D, Wang G, Nakaoka M, Lenz M (**submitted to Marine Biology**) In-situ-common-garden-assays demonstrate increased chemical defence against natural fouling in non-native populations of an aquatic organism.

ML, FW and SW conceived and designed the experiments. SW performed the experiments and collected algal material. GW, DB and MN contributed to algal collection and the places in China and Japan for bioassays. ML, FW and SW performed statistical analyses. SW wrote the manuscript, and ML and FW contributed to revisions.

Paper III

Wang S, Weinberger F, Lenz M (**submitted to Biofouling**) Seasonal variation in antifouling activity of the red macroalga *Gracilaria vermiculophylla* from the Baltic Sea.

ML, FW and SW conceived and designed the experiments. SW performed the experiments and collected algal material. FW, ML and SW performed statistical analyses. SW wrote the manuscript, and FW and ML contributed to revisions.

Paper I

Journal of Ecology, 2016
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Anti-epiphyte defences in the red seaweed *Gracilaria vermiculophylla*: non-native algae are better defended than their native conspecifics

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Summary

1. Epibiosis in the marine environment is a stressor that may determine invasion success in introduced species. Previous comparisons showed resistance to epibionts can be higher in non-native than in resident seaweed species, but we do not know whether it is an intrinsic trait of the non-natives or it has been acquired during the invasion process. To elucidate this question, a comparison between native and non-native populations of the same species is needed.

2. Resistance against two groups of epiphytes was assessed in living thalli and in artificial substrata coated with surface extracts, both gained from four Asian (native) and four European (non-native) populations of the red alga *Gracilaria vermiculophylla*. Two diatom species and two filamentous macroalgae were used as micro- and macro-epiphytes, and one of each type was collected in Asia, while the other came from Europe. Laboratory assays were done in both distributional ranges of *G. vermiculophylla* and in different seasons. We used *G. vermiculophylla* from four populations in each range and used a fully-crossed design with the factors a) ‘Origin of *Gracilaria*’, b) ‘Origin of epiphytes’, c) ‘Season’ and d) ‘Solvent used for extraction’.

3. Both groups of epiphytes, regardless of their origin, attached less to living thalli and to surface extracts from non-native *G. vermiculophylla*. Fewer diatoms attached to hexane-based extracts, while fewer *Ceramium* filaments settled on extracts gained with dichloromethane.

4. *Synthesis*. Our results show for the first time that non-native individuals of a marine organism are better defended against epiphytes than native conspecifics. Furthermore, we found evidence that at least a part of the defence is based on extractable secondary metabolites. We discuss several mechanisms that could explain the increased resistance to epiphytes in non-native individuals, including the release from enemies in the non-native range, which could lead to an increase in algal performance during the invasion process. We suggest that an enhanced defence against epiphytes after introduction is one reason for *G. vermiculophylla*’s invasion success. Our observation may also apply to other basibiont-epibiont and host-enemy systems, including plant-plant, plant-animal and animal-animal interactions, in aquatic environments and could be a key feature of bioinvasions.

Keywords: anti-fouling, biological invasions, chemical defence, Enemy Release Hypothesis, epiphytes, fouling, *Gracilaria vermiculophylla*, invasion ecology, non-native seaweeds

Introduction

Biological invasions are an important component of global change (Mack *et al.* 2000; Ricciardi 2007) and can cause severe ecological or economic problems by altering local biodiversity and affecting the services of ecosystems (Pimentel, Zuniga & Morrison 2005; Williams & Smith 2007; Vilà *et al.* 2011; Newton *et al.* 2013; Paini *et al.* 2016). Therefore, bioinvasions in the aquatic and terrestrial realm are receiving attention by scientists, authorities and environmental managers worldwide (Torchin & Mitchell 2004; Olenin *et al.* 2014). This growing awareness is needful, since the number of successful invasions is rising rapidly. In the marine environment, for instance, it increases exponentially and about 20% of the invasions are due to the spread of macroalgae (Schaffelke, Smith & Hewitt 2006).

To manage invasive species and to alleviate their negative effects, it is necessary to understand the mechanisms that determine the success or failure of invasions: Why do certain species invade new habitats successfully while others fail? Which factors allow the establishment and spread of introduced species? Currently, several hypotheses suggest explanatory concepts for these questions. Among them, the Enemy Release Hypothesis (ERH) is one of the most well-known (Keane & Crawley 2002). It states that when species are introduced into new habitats they are confronted with an abiotic and biotic environment that can be substantially different from the one they adapted to over evolutionary time scales. If co-evolved antagonists are absent in the new habitat and resident predators or parasites cannot recognise the newly introduced species as a resource, a release of the introduced species results from enemy control (Vermeij *et al.* 2009; Cacabelos *et al.* 2010). Furthermore, the Evolution of Increased Competitive Ability Hypothesis (EICA), which is an extension of the ERH, argues that non-native species that are released from their native enemies do not need to defend at all or, at least, less than in their home range. This release would allow them to invest more energy into growth, reproduction or into tolerating environmental stress (Hierro, Maron & Callaway 2005; Joshi & Vrieling 2005; Lenz *et al.* 2011), what, in turn, should make them competitively superior to native species (Blossey & Nötzold 1995; Müller-Schärer, Schaffner & Steinger 2004). Finally, the "Novel Weapons"-hypothesis by Callaway and Ridenour (2004) suggests that non-native species should be less susceptible to resident enemies than native ones, because they possess biochemical defence mechanisms to which native antagonists have not adapted to so far.

Most if not all marine organisms are prone to colonization by sessile life forms (epibionts), including bacteria, protists, microalgae, macroalgae and invertebrates. This phenomenon is known as epibiosis and it can severely impair the performance of the host organisms (basibionts) (Wahl 2008; Thomsen *et al.* 2012). It has been reported that epibionts can affect the growth and survival of macroalgal hosts by a) limiting the uptake of oxygen, carbon dioxide and nutrients through the thallus surface, b) reducing the amount of light available for photosynthesis, c) physically inhibiting sporulation, d) decreasing thallus flexibility, and/or e) increasing the palatability of the thallus (Wahl, Hay & Enderlein 1997; Hemmi *et al.* 2005). Macroalgae therefore need physical or chemical defences to minimize colonization of their surfaces.

All the hypotheses described above should also hold true for host-epibiont interactions, but have so far been widely neglected as a factor determining invasion success. So far, studies that tried to elucidate the relevance of host-enemy interactions for biological invasions mainly focused on non-native spermatophytes and their antagonists as well as their competitors in terrestrial systems (Keane & Crawley 2002; Verhoeven *et al.* 2009), while less research has been done on marine species (Wikström *et al.* 2006). Furthermore, most studies on non-native species - resident enemy interactions in marine systems considered herbivory (Wikström *et al.* 2006; Forslund, Wikström & Pavia 2010; Engelen *et al.* 2011; Hammann *et al.* 2013), while little is known about the interactions between non-native basibionts and resident epibionts (Strong, Maggs & Johnson 2009; Baer & Stengel 2014). A comparison between the non-native brown alga *Fucus evanescens* and the native *Fucus vesiculosus* in Swedish waters revealed that resistance to epibiosis was higher in the non-native species (Wikström & Kautsky 2004; Wikström & Pavia 2004). However, we lack knowledge whether this difference is based on species-specific traits or whether resistance to epibiosis can be gained during the invasion process. This could, for instance, be due to the release from other enemies that would allow the allocation of energy to defences against epibionts.

To elucidate whether non-native seaweed species are better defended against epibionts than their native conspecifics, we compared the susceptibility to epiphytism by microalgae and macroalgae between native and non-native populations of the red macroalga *Gracilaria vermiculophylla*. This perennial seaweed originates from the Northwest Pacific but during the last four decades it invaded many coastal habitats in the eastern Pacific (Bellorin, Oliveira & Oliveira 2004), the eastern Atlantic (Rueness 2005) and the western Atlantic (Freshwater *et al.* 2006; Thomsen *et al.* 2006) and the Mediterranean Sea (Sfriso *et al.* 2012). *Gracilaria vermiculophylla* has proven to be a particularly suitable marine model organism for the testing of theoretical concepts that predict the causes or consequences of biological invasions. It has, for example, been demonstrated that non-native populations of this species have a much lower genetic diversity than native populations (Kim, Weinberger & Boo 2010), while, nonetheless, the former proved to be more resistant towards herbivory (Weinberger *et al.* 2008; Rempt *et al.* 2012; Hammann *et al.* 2013; Hammann *et al.* 2016) and heat stress (Hammann 2014). These findings shed light on potential reasons for the invasion success of *G. vermiculophylla* and stimulated curiosity about how this seaweed interacts with epibionts in its non-native range.

Putative differences in the resistance against micro-epiphytes between native and non-native populations of *G. vermiculophylla* have been studied for bacteria (Saha *et al.* 2016), while we are not aware of studies that were done with eukaryote micro-epiphytes and macro-epiphytes. We conducted common garden experiments, i.e. individuals of *G.*

vermiculophylla from different geographic ranges were transplanted into a common environment, with *G. vermiculophylla* from Asia and Europe and with micro- and macro-epiphytes coming from the macroalga's native and non-native range.

Materials and methods

Collection of *Gracilaria vermiculophylla* and of epiphytes

Algal individuals were collected from four native and four non-native populations (Table 1). Sampling was conducted three times: from May to June and again from August to September 2014 as well as from June to July 2015. Laboratory experiments were conducted in June (summer) and in October (autumn) 2014 at the Helmholtz Centre for Ocean Research at Kiel, Germany, and in September (autumn) 2014 at the Xunshan Group Co., Ltd, Rongcheng, China, as well as in July (summer) 2015 at the Akkeshi Marine Station, Akkeshi, Japan (Table 2). Prior to experiments, living algal specimens from all sampling sites were transferred to the respective laboratory and kept under laboratory conditions for at least one week to allow them to recover from the transport (see Appendix S1 in Supporting Information for details).

Two pennate diatom species of the genus *Stauroneis* were isolated from individuals of *G. vermiculophylla* that were collected in Rongcheng and in the Kiel Fjord, respectively. Individuals of *Ceramium tenerrimum* were collected in Rongcheng, while specimens of *Ceramium virgatum* stem from Kiel Fjord (see Appendix S2 for details).

Algal surface extraction

Before the extraction procedure, adherent water was removed from the algal thalli in a centrifuge (Eppendorf 5810 R) with 200 rpm for 30 s. Surface associated metabolites were extracted from 80 g algal wet mass using the 'dipping technique' (de Nys, Dworjanyn & Steinberg 1998; Nylund *et al.* 2007), by dipping the algal individuals into a stirred mixture of dichloromethane (DCM) and hexane 1:4 (v/v) for 5 s. This procedure was benign and was chosen after different mixtures of solvents and dipping times were tested with regard to their effects on the survival of epidermal cells which was verified with Evan's blue (Figure S1). This was done to make sure that any damaging of cell walls, which could have led to the leaching of non-surface compounds, was avoided. The resulting solution was immediately filtered through a paper filter (Macherey Nagel, 185 mm in diameter) to remove particles and

Table 1. Sampling information of the four native and four non-native populations of *Gracilaria vermiculophylla*.

Origin	Collection site	Geographic Coordinate	First sampling	Second sampling	Third sampling	Salinity	Attachm-ent status	Morphology	Epibionts (incomplete information)
Native	Rongcheng, China, Yellow Sea	37°9'4.29"N, 122°33'35.60"E	21.05.2014	23.09.2014	06.07.2015	28-33	Holdfast	Brown, relatively more branches	Diatoms, *Ceramium tenerrimum*, Ulva sp., *Polysiphonia* sp., *Folliculina* sp.
	Qingdao, China, Yellow Sea	36°3'0.6"N, 120°20'59.1"E	21.05.2014	21.09.2014	06.07.2015	28-33	Holdfast	Brown, relatively more branches	Diatoms, *Ceramium tenerrimum*, Ulva sp., *Cladophora* sp.
	Akkeshi, Japan, Northeastern Honshu	43°1'25.80"N, 144°52'47.20"E	01.05.2014	19.08.2014	16.07.2015	29-33	Holdfast	Brown, relatively more branches	Diatoms, *Ceramium kondoi*, *Circeis spirillum*
	Tokyo, Japan, Central Kuroshio Current	35°19'25.72"N, 139°38'8.30"E	01.06.2014	02.08.2014	10.07.2015	29-33	Holdfast	Brown, relatively more branches	Diatoms, Ulva sp.
Non-native	Kiel, Germany, Baltic Sea	54°23'57.03"N, 10°12'53.12"E	02.06.2014	12.08.2014	12.07.2015	15-20	Free-floating	dark brown, relatively fewer branches	Diatoms, *Ceramium tenuicorne*, Ulva sp., *Pilayella* sp., *Amphibalanus improvises*, *Mytilus edulis*
	Nordstrand, Germany, North Sea	54°29'10.0"N 8°48'44.8"E	25.05.2014	04.09.2014	08.07.2015	30-32	Free-floating	dark brown, relatively fewer branches	Diatoms, Ulva sp. *Chaetomorpha* sp., *Cladophora* sp., *Amphibalanus improvises*, *Mytilus edulis*
	Belon, France, South European Atlantic Shelf	47°49'35.80"N, 3°40'20.50"W	18.05.2014	08.09.2014		2-33	Free-floating	dark brown, relatively fewer branches	Diatoms
	Pouldouran, France, Celtic Seas	48°45'55.90"N, 3°12'1.40"W	18.05.2014	08.09.2014	18.06.2015	2-33	Free-floating	dark brown, relatively fewer branches	Diatoms

Holdfast: thalli attached by a holdfast to the substratum; Free-floating: thalli not attached by a holdfast.

Table 2. Overview of the locations and timing over the attachment assays with *Gracilaria vermiculophylla* and different epiphytes.

Epiphytes	Assays after first sampling	Assays after second sampling	Assays after third sampling
Diatoms from Rongcheng	Kiel, 11.06.2014	Kiel, 24.10.2014	Akkeshi, 20.07.2015
Diatoms from Kiel	Kiel, 11.06.2014	Kiel, 24.10.2014	
Ceramium tenerrimum **from Rongcheng**	Rongcheng, 30.09.2014	Rongcheng, 30.09.2014	
Ceramium virgatum **from Kiel**	Kiel, 10.06.2014	Kiel, 23.10.2014	

the solvents were then evaporated under vacuum at 30 °C. The residue was re-dissolved in hexane to exclude non-polar compounds and this step was repeated until the hexane appeared colourless. The residue that remained after this first extraction step was then re-dissolved in DCM to extract existing polar components. Finally, 4 ml of both extracts were collected and stored at -20 °C.

Extracted surface area

To identify the extracted surface area, the relationship between algal surface area and algal wet weight was determined. Ten algal fragments, taken haphazardly across all populations, were carefully dried with paper and then scanned and weighed. The imaging software Image J (National Institute of Health, Bethesda, Maryland, USA) was used to analyze the surface area of each fragment. The algal thallus was viewed as a cylinder, so the projection area = thallus diameter × thallus length. The surface would then be = π × thallus diameter × thallus length = π × projection area. We identified the average surface area per g algal material across all ten fragments as 46.06 ± 2.8 $cm^2 g^{-1}$ (mean ± SD). The total extracted surface area was 80 g * 46.06 ± 2.8 $cm^2 g^{-1}$ = 3684.8 ± 224 cm^2.

Defence capacity against diatoms in living *Gracilaria vermiculophylla* thalli

We combined living thalli of *G. vermiculophylla* from both ranges (native and non-native) with diatoms from Kiel as well as Rongcheng, respectively. For each basibiont-epibiont combination, six fragments of 2 cm were cut from six randomly chosen algal individuals (n = 6) and were placed separately into the wells of a 6-well plate (Standard, Sarstedt AG &

Co.) together with 3 ml of a homogenized diatom suspension per well. The covered plate was then incubated for 3 h. A pilot study was done with different incubation periods (3 h, 5 h and 6 h), but diatom attachment did not increase any further after 3 h (Table S1). During incubation the plates were placed on a rotary shaker (100 rpm) that served to gently homogenize the suspension. Afterwards, each algal fragment was rinsed with 3 ml of sterile seawater. Algal pieces were then transferred to tubes containing 50 ml of sterile seawater and all attached diatoms were extricated by shaking the tube with a vortex shaker for 3 min. The shaken-off diatoms were collected on polycarbonate filters (0.2 µm pore size, 25 mm in diameter), which were then inspected under a fluorescence microscope and photographed. The photos were later used to assess the number of diatoms per algal fragment. The tested algal area was determined by scanning the fragments afterwards and quantifying their surface area with Image J.

Defence capacity against *Ceramium* sp. in living *Gracilaria vermiculophylla* thalli

The majority of studies about the attachment of epiphytes or epizoans to hosts focused on colonization by microscopic propagules and spores, which are the mobile stages of the otherwise sessile epibionts. However, vegetative thalli of *Ceramium* sp. can directly attach or penetrate into the host by the formation of hapteria (Leonardi *et al.* 2006; Lion *et al.* 2006; Michetti *et al.* 2016). We therefore conducted assays with *C. tenerrimum* and *C. virgatum* with filaments of these algae and organized them in the same way as the diatom trials described in the previous paragraph. For this, ten algal individuals per population of *G. vermiculophylla* were used (i.e. n = 10 per basibiont-epibiont combination). From each of these ten replicates we cut a fragment of 2 cm, while a *Ceramium* filament of the same length was then bound to *G. vermiculophylla* using colored paper clips. This was done to shorten the distance between the fragments and by this to increase the likelihood of attachment. These pairs were put into Petri dishes containing 30 ml of the modified culture medium of Provasoli’s enriched seawater (PES) (Bold & Wynne 1978). The covered Petri dishes were incubated for two weeks and attachment rates were quantified after this time.

Chemical defence capacity against diatoms in *Gracilaria vermiculophylla* surface extracts

These assays were organized in the same way as the ones with living thalli. For the assays with extracts we used an extract concentration that was five times higher than the natural surface concentration. This was done to compensate for the possible degradation and incomplete extraction of active compounds. In one cylindrical well of a 96-well plate (flat bottom, Greiner bio-one), 100 µl of both, DCM and hexane, cover a total surface area of 94 mm². Thus, 5.1 µl of surface extracts and 94.9 µl of pure solvent were then pipetted into each well to cover the aspired wall area. Wells loaded with pure DCM and hexane were later used as controls. Solvents were then evaporated overnight in a freeze-dryer. After this, 100 µl of the homogenized diatom suspension were transferred into the wells. Four wells of each experimental group received extracts and diatoms, while four wells received extracts only to check for extract background fluorescence. Control wells were treated in the same way. Afterwards, the covered 96-well plate was incubated for 3 h and then each well was rinsed with 200 µl of sterile seawater. Finally, fluorescence intensity per well was measured and the number of diatoms per well was calculated from fluorescence intensity by using the linear function that was established in a pilot study (Appendix S3, Figure S2).

Chemical defence capacity against *Ceramium* sp. in *Gracilaria vermiculophylla* surface extracts

These assays were organized in the same way as the ones with living thalli. In a 6-well plate, 120 µl of solvent can cover the total surface area of the bottom of one well. A paper filter (Carl Roth, 3.5 cm in diameter) was put into each well to avoid erosion by solvents. For applying a fivefold natural surface concentration, 52 µl of surface extracts and 68 µl of pure solvent were then pipetted into each well, while we had five wells per population. Some wells received pure DCM or hexane and served as controls. The solvent was then evaporated overnight in a freeze-dryer. After that, 5 ml of PES medium and ten *Ceramium* filaments (1 cm) were transferred to each well. The covered 6-well plate was then incubated for two weeks. Afterwards, the proportion of *Ceramium* filaments that attached to the paper filter was quantified.

Statistical analyses

All statistical and graphical analyses were done using the free statistical computing software R (R Development Core Team 2014). We used mixed effect-modelling to analyze the data from our multifactorial experimental approach. In case of the assays with living thalli, our experimental design included three fixed factors: 1) ‘Origin’ with the levels ‘Native’ and ‘Non-native’ (i.e. origin of *Gracilaria*), 2) ‘Diatom’ / ‘*Ceramium*’ with the levels ‘China’ and ‘Germany’ (i.e. origin of epiphytes), and 3) ‘Season’ with the levels ‘Summer’ and ‘Autumn’ (i.e. the time of the experiment). In the assays with surface extracts we had one more fixed factor: ‘Solvent’ with the levels ‘DCM’ and ‘hexane’. In all analyses, the algal sampling sites were included as a random factor, while the two types of epiphytes (diatoms/*Ceramium*) were analyzed separately. To achieve homogeneity of variances and normality of errors, data from the assays with diatoms and living thalli were square root transformed and data from the assays with diatoms and surface extracts were log-transformed. However, homogeneity of variances could not be achieved for all factors. We therefore included weights for ‘Season’ and ‘Diatom’ to account for the differences in the variance structures between their factor levels in the modelling. For this we used the varIdent function of the nlme package in R (Zuur *et al.* 2009). Test assumptions were checked graphically with residual plots (Zuur, Ieno & Elphick 2010). In addition, a mixed effect-modelling, with the factors ‘Origin’, ‘Diatom’ / ‘*Ceramium*’, ‘Season’ and ‘Material’(i.e. thalli and surface extracts), was used to analyze the data from the two assays with diatoms and from the two assays with *Ceramium* sp., respectively, in a common approach (see results in Table S2, S3). To achieve homogeneity of variances and normality of errors, data from the two assays with diatoms were square root transformed.

Results

Defence capacity against diatoms in living *Gracilaria vermiculophylla* thalli

After 3 h of exposure to colonization by diatoms, a three-way-interaction among the factors ‘Diatom’, ‘Origin’ and ‘Season’ was observed: Fewer diatoms from Rongcheng attached to non-native than to native *G. vermiculophylla* in autumn, and this difference was less pronounced in Summer and less observed with diatoms from Kiel in both seasons (Fig. 1, Table 3). For both diatom species, fewer cells (by 60% less) attached to non-native than to native *G. vermiculophylla* individuals (Fig. 1, Table 3) and only 4% of the unexplained variation was found to be covered by the random factor ‘Site’. Furthermore, for both diatom species, settlement rates were on average by 66% lower in summer than in autumn.

Table 3. Influence of season, origin of *Gracilaria vermiculophylla* and origin of diatoms on the attachment rates of diatoms on living thalli. Results from mixed-effect modelling.

Source of variation	numDF	denDF	F - value	p - value
Season	1	220	283.691	<.0001
Origin	1	6	138.724	<.0001
Diatom	1	220	24.636	<.0001
Diatom:Origin	1	220	4.429	0.0365
Diatom:Season	1	220	2.047	0.1540
Origin:Season	1	220	40.930	<.0001
Diatom:Origin:Season	1	220	11.439	0.0009

Averaged across the two seasons, diatoms from Kiel settled by 21% less often on *G. vermiculophylla* thalli than their congeners from Rongcheng (Fig. 1, Table 3).

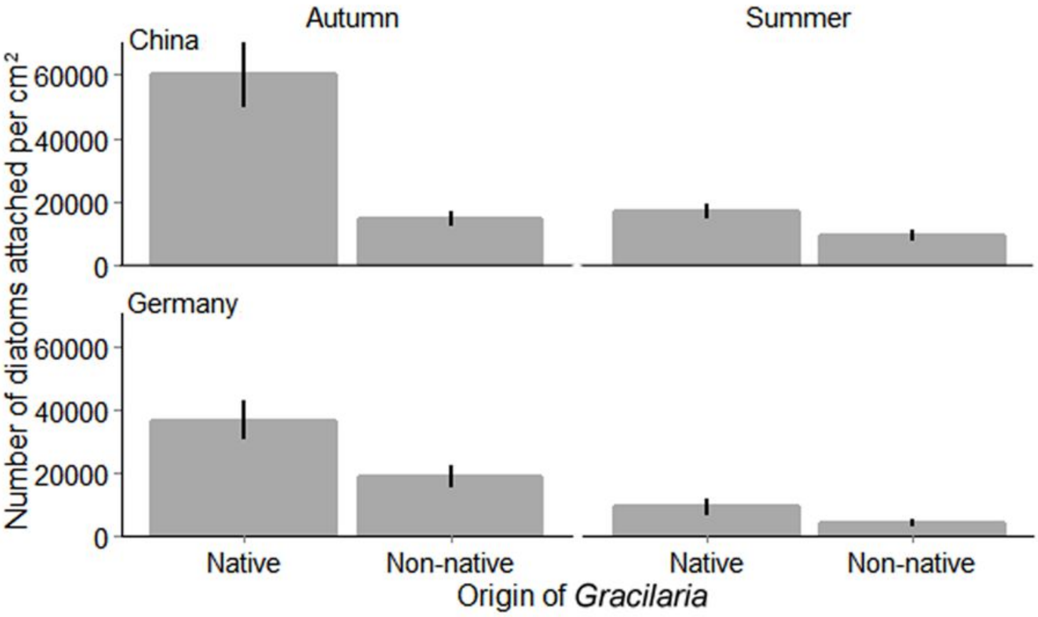

Fig. 1. Colonization of living thalli of native and non-native *Gracilaria vermiculophylla* by diatoms from both origins. Assays were run in summer and autumn 2014 and in summer 2015. Means and 95% CIs, n = 24–48 in each group.

Defence capacity against *Ceramium* sp. in living *Gracilaria vermiculophylla* thalli

After two weeks of colonization by *Ceramium* filaments there was an interaction between '*Ceramium*' and 'Origin' (Fig. 2, Table 4). *Ceramium virgatum* (from Kiel, Germany) attached less to non-native than to native *G. vermiculophylla*, while this difference was less pronounced in *C. tenerrimum* (from Rongcheng, China). An interaction between '*Ceramium*' and 'Season' also emerged since attachment rates of *C. tenerrimum* differed between autumn and summer, while this was not the case for *C. virgatum* (Fig. 2, Table 4). Filaments of both *Ceramium* species attached, on average, by 33% less often to non-native than to native

Table 4. Influence of season, origin of *Gracilaria vermiculophylla* and origin of *Ceramium* on filament attachment rates on living thalli. Results from mixed effect-modelling.

Source of variation	numDF	denDF	F - value	p - value
Season	1	20	8.4325	0.0088
Origin	1	6	30.5134	0.0015
Ceramium	1	20	2.1081	0.1620
***Ceramium*:Origin**	1	20	13.1757	0.0017
***Ceramium*:Season**	1	20	75.8923	<.0001
Origin:Season	1	18	2.2500	0.1510
***Ceramium*:Origin:Season**	1	18	0.5625	0.4629

G.vermiculophylla specimens (Fig. 2, Table 4). In this case, 37% of the unexplained variation was covered by ‘Site’. Furthermore, attachment rates, averaged across both *Ceramium* species, were by 10% lower in autumn than in summer, while they, when averaged across both seasons, did not differ between the two *Ceramium* species (Fig. 2, Table 4).

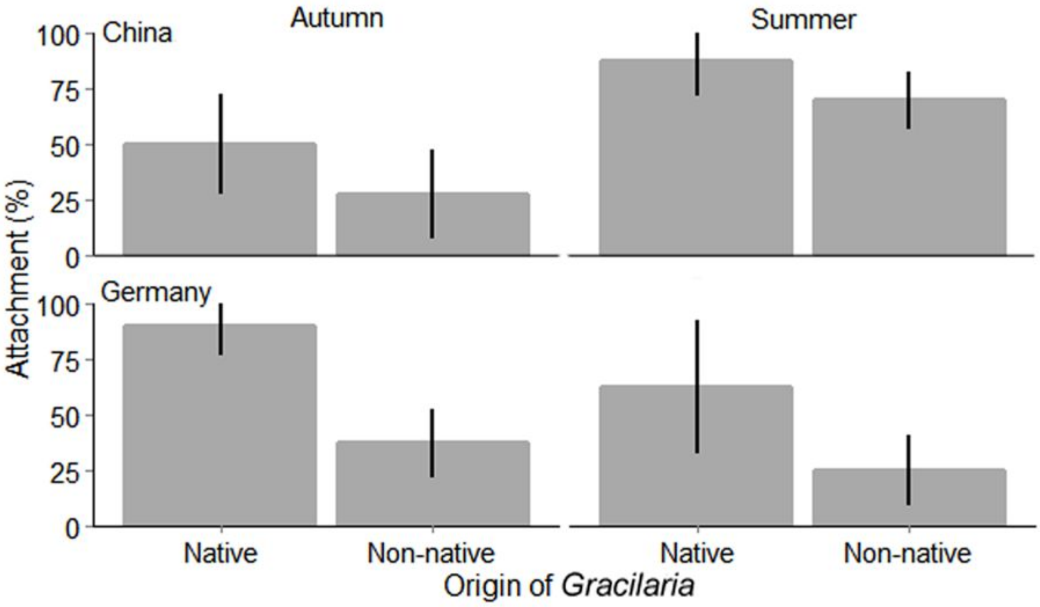

Fig. 2. Colonization of living thalli of native and non-native *Gracilaria vermiculophylla* by *Ceramium* from both origins, *C. virgatum* from Germany and *C. tenerrimum* from China. Assays were run in summer and autumn 2014. Means and 95% CIs, n = 40 in each group.

Chemical defence capacity against diatoms in *Gracilaria vermiculophylla* surface extracts

The results from this assay are generally in accordance with those of the diatom trials with living thalli. After exposing the surface extracts to diatom settlement for 3 h, an interaction among the factors ‘Solvent’, ‘Diatom’ and ‘Season’ was observed: Attachment rates of diatoms from Kiel on surfaces coated with DCM-based extracts were lower in summer than in autumn, but no such difference was observed on surfaces coated with hexane-based extracts or with diatoms from Rongcheng on any coated surfaces (Table 5). Additionally, fewer diatoms attached (by 9% less) to surfaces coated with extracts from non-native than from native *G. vermiculophylla* (Figs 3 and 4, Table 5) and only 2% of the unexplained variation was covered by ‘Site’. In general, diatom settlement rates were again by 22% lower in summer than in autumn (Figs 3 and 4, Table 5). Interestingly, different from the assays with living thalli, diatoms from Kiel settled two times more often than diatoms from Rongcheng (Fig. 3, Table 5). Moreover, we found fewer diatoms (by 4% less) attached to surfaces covered with non-polar compounds (extracted with hexane) than to those coated with polar compounds (extracted with DCM) (Fig. 4, Table 5).

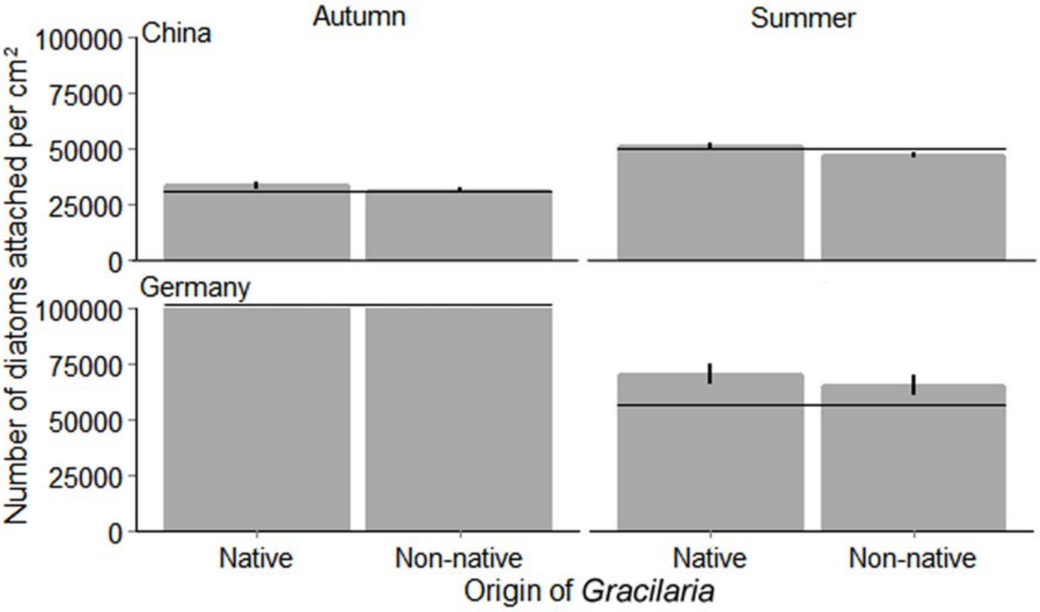

Fig. 3. Colonization of surface extracts from native and non-native *Gracilaria vermiculophylla* by diatoms from both origins. Assays were run in summer and autumn 2014. Means and 95% CIs, n = 32 in each group. The horizontal lines indicate mean colonization rate on controls, which were without extracts (n = 8).

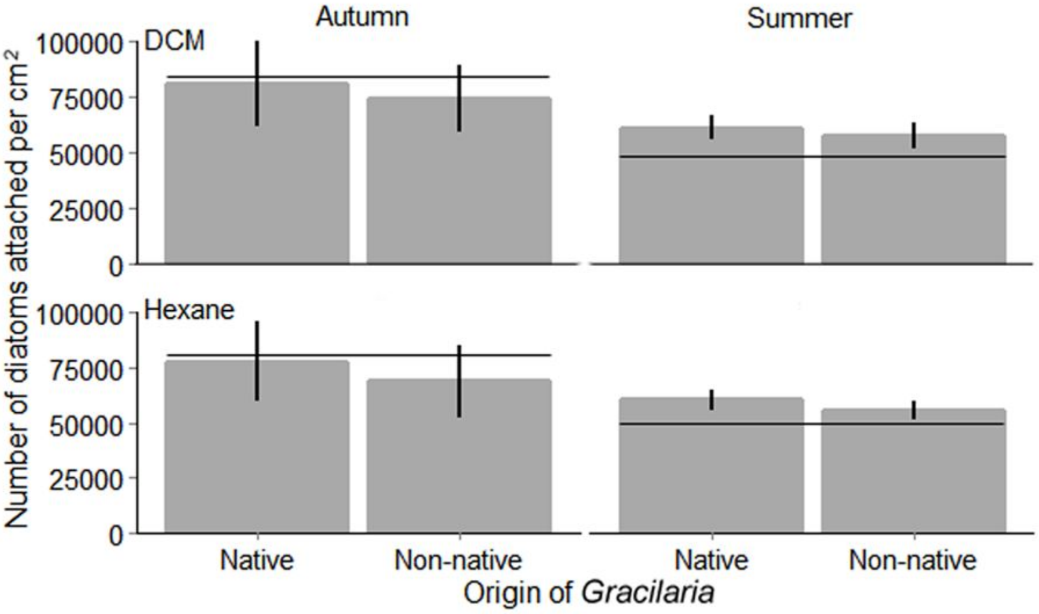

Fig. 4. Colonization of DCM and hexane surface extracts from native and non-native *Gracilaria vermiculophylla* by diatoms from both origins. Assays were run in summer and autumn 2014. Means and 95% CIs, n = 32 in each group. The horizontal lines indicate mean colonization rate on controls, which were without extracts (n = 8).

Table 5. Influence of season, solvent, origin of *Gracilaria vermiculophylla* and origin of diatoms on the attachment rates of diatoms on surface extracts. Results from mixed effect-modelling.

Source of variation	numDF	denDF	F - value	p - value
Season	1	238	283.0	<.0001
Solvent	1	238	17.4	<.0001
Origin	1	6	11.8	0.0139
Diatom	1	238	1772.5	<.0001
Solvent:Season	1	238	21.9	<.0001
Diatom:Season	1	238	648.9	<.0001
Solvent:Diatom	1	238	0.2	0.6686
Solvent:Origin	1	238	1.3	0.2479
Diatom:Origin	1	238	0.1	0.7242
Origin:Season	1	238	0.0	0.9599
Solvent:Diatom:Season	1	238	6.0	0.0154
Solvent:Diatom:Origin	1	234	0.1	0.8000
Solvent:Origin:Season	1	234	0.7	0.4059
Diatom:Origin:Season	1	234	0.3	0.6021
Solvent:Diatom:Origin:Season	1	234	0.0	0.9073

Chemical defence capacity against *Ceramium* sp. in *Gracilaria vermiculophylla* surface extracts

After two weeks of exposing surface extracts to colonization by *Ceramium* filaments, there was an interaction between '*Ceramium*' and 'Season': Attachment rates of *C. virgatum* were lower in autumn than in summer, while this difference was less pronounced in *C. tenerrimum* (Fig. 5, Table 6). In addition, in autumn we found that fewer (by 13% less) *Ceramium* filaments attached to surfaces coated with moderately polar compounds than on such covered with non-polar compounds. This was not the case in summer and this difference led to an interaction between 'Solvent' and 'Season' (Fig. 6, Table 6). Furthermore, fewer *Ceramium* filaments (by 10% less) attached to surfaces coated with extracts gained from non-native than to surfaces with extracts from native *G. vermiculophylla* (Figs 5 and 6, Table 6) and only 0.5% of the unexplained variation was covered by 'Site'. In general, *Ceramium* filaments attached 22% less often in autumn than in summer (Figs 5 and 6, Table 6), but attachment rates never differed between the two *Ceramium* species (Fig. 5, Table 6).

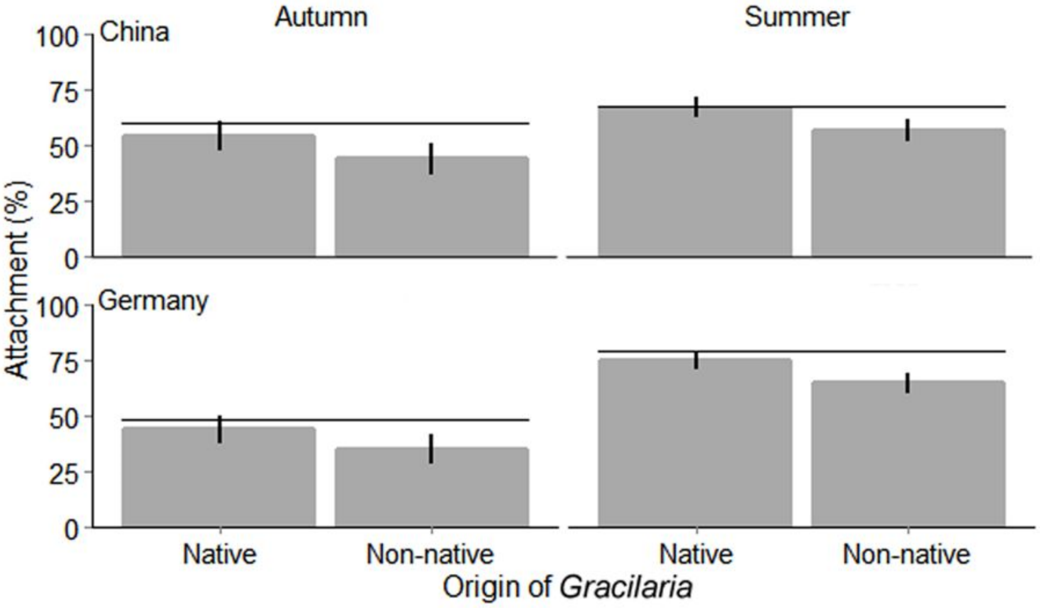

Fig. 5. Colonization of surface extracts from native and non-native *Gracilaria vermiculophylla* by *Ceramium* from both origins, *C. virgatum* from Germany and *C. tenerrimum* from China. Assays were run in summer and autumn 2014. Means and 95% CIs, n = 40 in each group. The horizontal lines indicate mean colonization rate on controls, which were without extracts (n = 10).

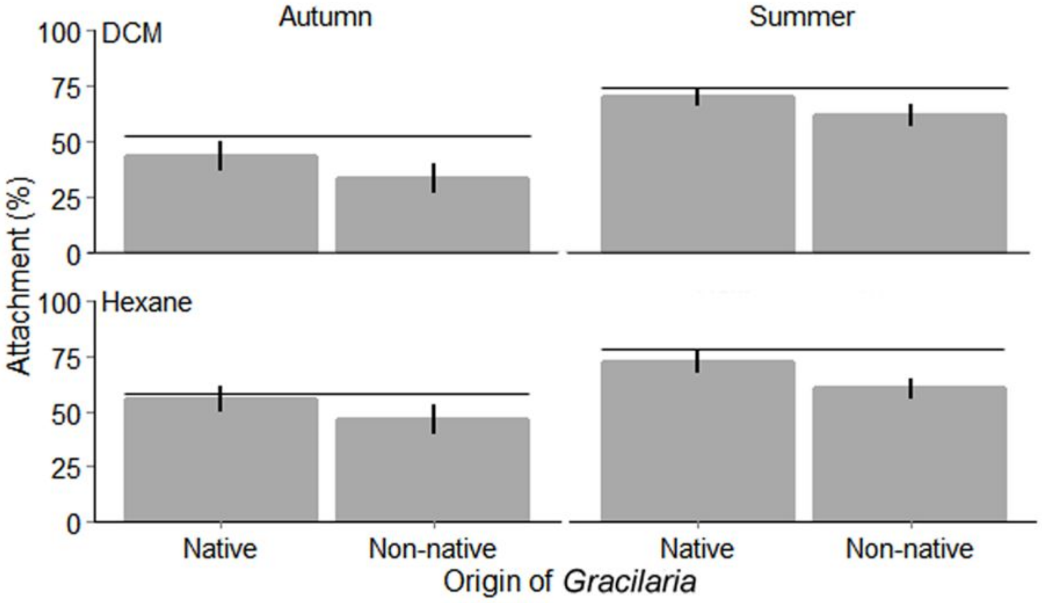

Fig. 6. Colonization of DCM and hexane surface extracts from native and non-native *Gracilaria vermiculophylla* by *Ceramium* from both origins, *C. virgatum* from Germany and *C. tenerrimum* from China. Assays were run in summer and autumn 2014. Means and 95% CIs, n = 40 in each group. The horizontal lines indicate mean colonization rate on controls, which were without extracts (n = 10).

Table 6. Influence of season, solvent, origin of *Gracilaria vermiculophylla* and origin of *Ceramium* on filament attachment rates on surface extracts. Results from mixed effect-modelling.

Source of variation	numDF	denDF	F - value	p - value
Season	1	307	124.418	<.0001
Solvent	1	307	11.090	0.0010
Origin	1	6	27.095	0.0020
Ceramium	1	307	0.126	0.7224
Solvent:Season	1	307	9.835	0.0019
***Ceramium*:Season**	1	307	20.782	<.0001
Solvent:*Ceramium*	1	298	2.308	0.1297
Solvent:Origin	1	298	0.126	0.7224
***Ceramium*:Origin**	1	298	0.051	0.8211
Origin:Season	1	298	0.026	0.8717
Solvent:*Ceramium*:Origin	1	298	0.001	0.9742
Solvent:*Ceramium*:Season	1	298	3.395	0.0664
Solvent:Origin:Season	1	298	0.235	0.6281
***Ceramium*:Origin:Season**	1	298	0.026	0.8717
Solvent:*Ceramium*:Origin:Season	1	298	2.718	0.1003

Discussion

For this intra-specific comparison, we sampled individuals of *G. vermiculophylla* at different sampling locations in either the native or the non-native range of the species. Within the respective ranges, the different sampling sites were located in nearly the same biogeographical region according to the Marine Ecoregions of the World (MEOW) system suggested by Spalding *et al*. (2007). The sites in the native range were located in the Cold Temperate Northwest Pacific/Yellow Sea (China) and in Northeastern Honshu (Japan) as well as in the Warm Temperate Northwest Pacific/Central Kuroshio Current (Japan), which is adjacent to Northeastern Honshu. The sites in the non-native range were located in the Northern European Sea/Baltic & North Sea (Germany) and in the Celtic Sea as well as the Lusitanian/South European Atlantic Shelf (France), which is adjacent to the Celtic Sea. Furthermore, we took care that the distances between the various sampling sites in the native as well as in the non-native range were similar, in order to have the same degree of between-site variability within the ranges. We therefore assumed that the within-range variability, which could be attributed to potential differences in the diversity and composition of the resident flora and fauna as well as to climate conditions, would be low. This assumption was confirmed by the low amount of unexplained variation (0.5% to 4%) that was actually covered by the random factor ‘Site’ in most of our statistical modellings. This was true for diatom attachment rates on both living thalli and on extracts and for *Ceramium* attachment to extracts. In contrast to this, *Ceramium* attachment rates on living thalli varied considerably

(37% of the unexplained variation) among sites within both ranges and we cannot plausibly explain this deviation from the otherwise consistent picture.

In our study we used two types of epiphytes to test for inter-population differences in *G. vermiculophylla* with regard to its susceptibility to epiphytes: Diatoms as a common type of micro-epiphytes and *Ceramium* filaments as a macro-epiphyte. We found that both of them, independent of their actual origin, attached by 60% and 33%, respectively, less to the living thalli of European *G. vermiculophylla* than to those of Asian conspecifics. In general, such a difference could either due to the fact that non-native *G. vermiculophylla* individuals are better defended and therefore attract fewer/repel more epiphytes or it could be due to lower settlement rates of native colonizers on the non-native macroalga. However, our experimental design excluded the latter option, since we exposed non-native macroalgae to epiphytes from the native as well as from the non-native range and both combinations showed the same trend. This finding indicates that non-native *G. vermiculophylla* are better defended against epiphytes than those that stem from the native range.

In macroalgae, resistance to epibiosis can be mediated by a) the thallus surface structure (Schumacher *et al.* 2007; Chapman *et al.* 2014), b) surface associated bacterial communities that repel epibionts (Boyd, Adams & Burgess 1999; Dobretsov, Dahms & Qian 2006), and c) surface-bound secondary metabolites that have anti-epibiont activities (Nylund *et al.* 2007; Saha *et al.* 2011; Thabard *et al.* 2011). The question is now whether one or more of these mechanisms changed with regard to their mode of action or with regard to their efficiency during the invasion process. Such a change could, *inter alia*, be caused by a directional selection of genotypes that exhibit a low susceptibility to epibionts during transport or after release into the new habitat. However, we cannot think of a scenario during these phases that would specifically select for resistance to epibiosis. Furthermore, since the non-native gene pool is a reduced subset of the gene pool in the donor region, it is possible, although presumably not very likely, that by chance an epibiosis-resistant genotype was highly frequent among the introduced individuals. Finally, a change in the quality of anti-epiphyte defences could be attributed to increased energy resources, which are a consequence of the release from abiotic and biotic pressures in the new environment (Joshi & Vrieling 2005). Under such conditions, non-native seaweeds may reduce specific defences they developed against enemies in their native range and shift energy resources towards more general anti-enemy defences. An observation made at our study site in the native range hints at the potential relevance of the last mechanism: In Rongcheng, China, an amphipod species, *Caprella* sp., is the main grazer of *G. vermiculophylla* in many habitats and it can consume substantial parts of this local alga during summer (S. Wang, pers. obs.). In Kiel, Germany, so far no herbivore makes use of this alga to such an extent and it seems that the grazing pressure on *G. vermiculophylla* is generally lower than in Rongcheng (Hammann *et al.* 2013). However, we do not have information whether the picture is the same in the other non-native habitats that we sampled in Europe. If *G. vermiculophylla* is mostly ungrazed in coastal habitats in Europe, this could have allowed the non-native *G. vermiculophylla* to allocate a larger part of their energy budget to anti-epibiont defences and this possibly caused their lower attractiveness for colonizers.

An important aspect of our study was to identify properties of *G. vermiculophylla* that mediate its anti-epiphyte defences. For this we compared epiphyte attachment rates on living thalli to those on extract coated surfaces. Here we observed that the general trend in epiphyte attachment was the same for living thalli and extract coated substrata. However, the effect size, i.e. the difference in the susceptibility to epiphytes in native and non-native *G. vermiculophylla*, was consistently smaller in the latter assays: Fewer diatoms (on average 9% for extracts, 60% for living thalli) and fewer *Ceramium* filaments (on average 10% for extracts, 33% for living thalli) attached to substrata that were covered with extracts from European *G. vermiculophylla* than to those with extracts from Asian specimens. This, first of all, confirms that resistance to epiphytes in *G. vermiculophylla* has, at least partly, a chemical component. If the lower susceptibility in non-native *G. vermiculophylla* is due to this chemical component, it could either be based on an increased synthesis of active compounds (Forslund, Wikström & Pavia 2010) or due to the presence of some chemical compounds that are novel to resident enemies in these individuals (Enge *et al.* 2012). Overall, extracts exhibited a lower inhibitory activity against diatom and *Ceramium* settlement than living algae. This difference could be due to the fact that active metabolites were insufficiently captured by the extraction process or degraded after extraction. Alternatively, other non-chemical components such as surface properties - which were of course excluded in the assays with extracts - could also have contributed to the overall deterrence. Finally, the compounds which were responsible for the anti-epiphyte activity we observed in living *G. vermiculophylla* may not only have stemmed from the thallus surface but also from the inside of algal cells. This reason could have been relevant since some epibionts, including species of the genus *Ceramium*, penetrate into algal thalli and therefore also get in contact with their interior (e.g. Leonardi *et al.* 2006). We have no data that could elucidate which of the three scenarios was responsible for the picture we observed. However, since we used only two solvents for the extractions (i.e. hexane and DCM) that cover a limited part of the polarity spectrum, it is at least likely that we missed relevant compounds and thereby underestimated the potential of chemical defences in *G. vermiculophylla*.

So far, no tests have been made to investigate whether the surface texture, microtopography or consistency of *G. vermiculophylla* thalli mediates a defence against epibionts. Such effects are known from *Saccharina* species (Chapman *et al.* 2014; da Gama, Plouguerné & Pereira 2014) that belong to the brown macroalgae and possess an outer cell wall with a mucilage consisting of alginic acid with traces of sulphated fucoidan that could, theoretically, act as a low-adhesion, gelatinous covering.

Whatever the mechanism is, a low susceptibility to epiphytes in non-native populations of *G. vermiculophylla* can, at least partly, explain the invasion success of the species. It has been proposed that marine algal invaders have more effective anti-epibiont defences than comparable resident species, e.g. in its non-native habitats in northern Europe the brown alga *Fucus evanescens* is known to get less colonized by filamentous algae and sessile invertebrates than its native congener *Fucus vesiculosus* (Wikström & Pavia 2004). When their surface is free of epibionts, macroalgae can take up more oxygen, carbon dioxide and nutrients. Furthermore, they receive more light for photosynthesis and are less prone to dislodgement caused by biomechanical drag. Furthermore, they may be less attractive to grazers (Wahl, Hay & Enderlein 1997). Therefore, algae, which are free of epibionts or show low degrees of epiphyte or epizoan cover, should have more energy available for reproduction and growth, as well as for tolerating adverse environmental conditions – what in turn should increase their potential to establish and spread in new environments.

The Enemy Release Hypothesis proposes that non-indigenous species are commonly released from biotic pressures, e.g. grazing, in their recipient habitat, because they leave their co-evolved antagonists behind while, at the same time, resident enemies fail to recognize the new species as a food source (Keane & Crawley 2002). In this context, Hammann *et al.* (2013) found that the periwinkle species *Littorina brevicula,* which lives in the native range of *G. vermiculophylla,* consumes more of this seaweed, regardless from which distributional range the algal material stems, than *Littorina littorea*, which is from its non-native range. This finding is presumably due to the fact that *L. brevicula* coevolved with *G. vermiculophylla* and can make better use of it as a food source. A somewhat comparable preference of native over non-native *G. vermiculophylla* was also shown by the diatoms we used in the assays with living thalli: diatoms from Rongcheng generally attached in higher numbers to *G. vermiculophylla* from both distributional ranges than diatoms from Kiel. This finding may also be due to the fact that these diatoms recognize *G. vermiculophylla* more readily as a suitable settlement substratum than diatoms from the non-native range. Surprisingly, we observed the opposite picture when we tested the surface extracts. This mismatch suggests that, besides a chemical defence, other properties of the thallus surface, such as its structure or the presence of biofilms, play a role in the anti-diatom defence in living thalli. However, we did not find a difference between the attachment rates exhibited by the two *Ceramium* species for both substrata. However, it is not clear why this epiphyte did not show a preference. It is possible that *C. virgatum* – with regard to *G. vermiculophylla* as a settlement substratum - generally has the same settlement capacity as *C. tenerrimum*. The absence of a difference is somewhat surprising, because the way the *Ceramium* filaments attach to algal surfaces constitutes a very intimate connection of the two organisms. It is most often characterized as an infection of the basibiont, because it is mediated by the formation of hapteria that first attach to the thalli of the host and then penetrate into its tissue (Lion *et al.* 2006). Lion *et al*. (2006) found that after wounding *Gracilaria chilensis* released oxylipins, which suppressed the development of hapteria in *Ceramium rubrum*. This fact indicates that co-evolution occurs between *Ceramium* species and their hosts and hence a difference in settlement rates between *C. tenerrimum* and *C. virgatum* would be likely.

Previous studies have suggested that season (Culioli *et al.* 2002; Hellio *et al.* 2004) can influence the capacity of a seaweed to defend itself against epibionts. In accordance with this finding, we found differences in epiphyte settlement rates between summer and autumn of the same year of which we assume that they are attributed to differences in the anti-epiphyte activity of *G. vermiculophylla*. Fewer diatoms attached to both living fragments and extract-coated surfaces in summer, while fewer *Ceramium* filaments attached to those substrates in autumn. Such inter-seasonal differences in anti-epiphyte defences presumably correlate with natural fluctuations in the overall propagule abundance in the colonizer pool (Steinberg & Vanaltena 1992; Amade & Lemée 1998; Wahl *et al.* 2010; Rickert *et al.* 2015), which means that marine macroalgae can adjust their anti-epibiont activities to quantitative or qualitative changes in colonization pressure. In both regions where we collected algae, diatoms are more abundant from April to June than from August to October (Trimonis, Vaikutiene & Gulbinskas 2010; Wang *et al.* 2014), while *Ceramium* is more abundant during the latter time span (Weinberger *et al.* 2014; S. Wang, pers. obs.). However, we collected our data only during the course of one year and we therefore do not have robust evidence for seasonality in the defence capacity of *G. vermiculophylla*. To establish such a pattern, assays would need to be repeated over several years.

A further interesting observation that we made was that fewer diatoms attached to surfaces covered with non-polar compounds than to those coated with polar compounds, while the opposite was true for *Ceramium* filaments. This indicates that the defences against these two epiphytes are mediated by compounds that differ in polarity. A similar observation has been reported earlier: surface compounds extracted with a mixture of hexane and DCM from *Caulerpa filiformis* significantly inhibited spore settlement of *Polysiphonia* sp., while more polar compounds, which were extracted with DCM from surfaces of the same species, inhibited settlement and germling development of gametes of *Ulva australis* (Nylund *et al.* 2007).

Our study is the second biogeographical comparison of defence capacities against epibionts between native and non-native populations of *G. vermiculophylla* (Saha *et al.* 2016), which is now invasive in many coastal areas worldwide. However, it gives the first evidence that the capacity to defend against epibionts is higher in non-native individuals than in native – regardless of whether the epibionts originate from the native of the non-native range of *G. vermiculophylla*. Our findings therefore seemingly contradict the observations made by Saha *et al*. (2016), who focused on seaweed-bacteria interactions and showed that non-native *G. vermiculophylla* are better defended against bacterial epibionts from the non-native range but, at the same time, have reduced their capacity to defend themselves against epibionts from their home range. The contradiction may be due to the use of different micro-epibionts. Bacteria are the first colonizers of bare substrata in the marine environment (Wahl 1989) and can regulate the production of bioactive compounds, motility, and biofilm formation by Quorum Sensing (QS), which is a density-dependent cell-cell signaling communication among bacteria (da Gama, Plouguerné & Pereira 2014). Furthermore, it is known that bacterial biofilm formation can mediate further colonization by eukaryote micro- and macro-epibionts. The differences between epibacteria and other epibionts could have led to the evolution of different defence strategies against them in seaweeds. Unlike compounds that function against eukaryote micro- and macro-epibionts through growth inhibition or lethality, most antimicrobial settlement and attachment defences impact the behavior of bacteria, such as swarming (Rasmussen & Givskov 2006).

Even though our study focused on macrophyte-epiphyte interactions in the marine environment, our findings should be applicable to all basibiont-epibiont interactions in aquatic systems, including plant-plant, plant-animal and animal-animal combinations, since epibionts are widespread and most of them are generalists (Wahl & Mark 1999). Additionally, our findings may also be applicable to host-herbivore interactions in aquatic systems, since, similar to epibionts, many herbivores are generalists and an increased chemical resistance to herbivory has already been documented in non-native plants and seaweeds (Forslund, Wikström & Pavia 2010).

We conclude that the lower susceptibility to epiphytes that we observed in non-native *G. vermiculophylla* cannot be explained by a lower epibiont pressure experienced by the non-native individuals, but is due to an elevated resistance to epibiosis that, at least partly, is linked to an enhanced chemical defence capacity. Our study therefore provides the first evidence of an increased resistance to epibiosis in introduced populations of a widely distributed marine species. This change in its performance during the invasion process may be critical for the invasion success of the macroalga.

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Supplementary information

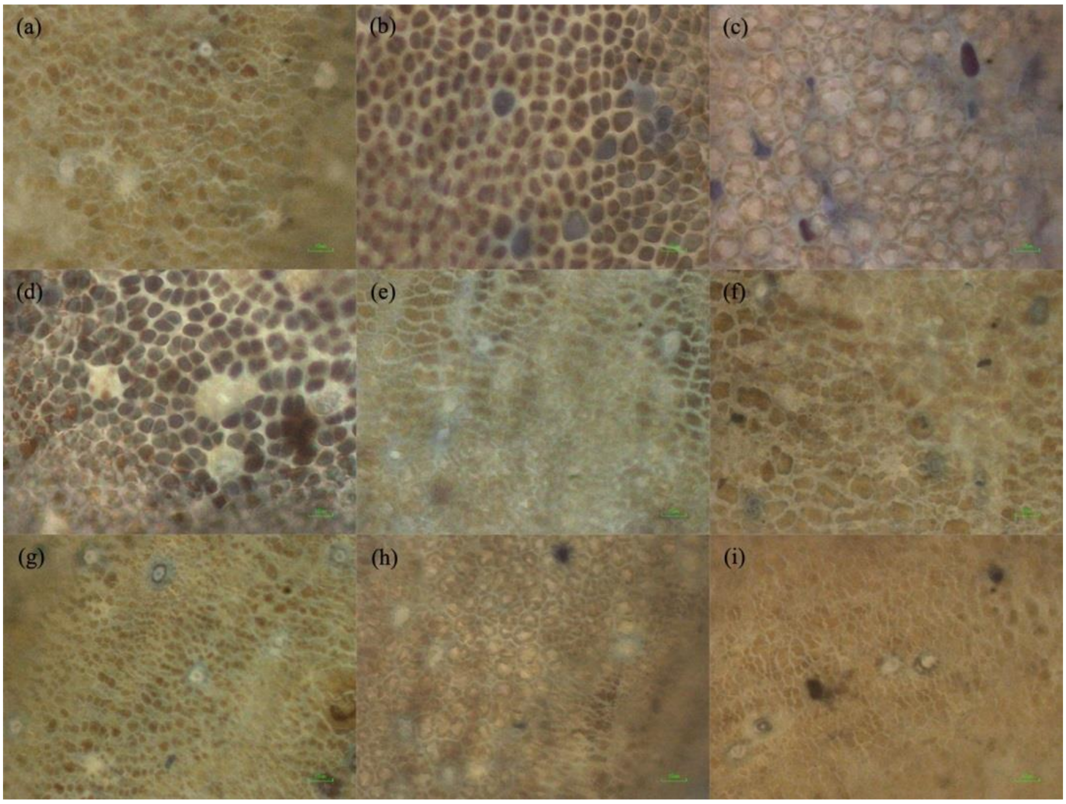

Figure S1. Determination of solvents and dipping times for surface extraction of *Gracilaria vermiculophylla*. (a) Healthy algal cells. The alga was extracted by: (b) Methanol-hexane mixture 1:9 (v/v) for 5 s. (c) Methanol-hexane mixture 1:19 (v/v) for 5 s. (d) Propanol-hexane mixture 1:9 (v/v) for 5 s. (e) Propanol-hexane mixture 1:19 (v/v) for 5 s. (f) dichloromethane (DCM)-hexane mixture 1:3 (v/v) for 5 s. (g) DCM-hexane mixture 1:4 (v/v) for 10 s. (h) DCM-hexane mixture 1:4 (v/v) for 7 s. (i) DCM-hexane mixture 1:4 (v/v) for 5 s. Scale bars: 10 µm.

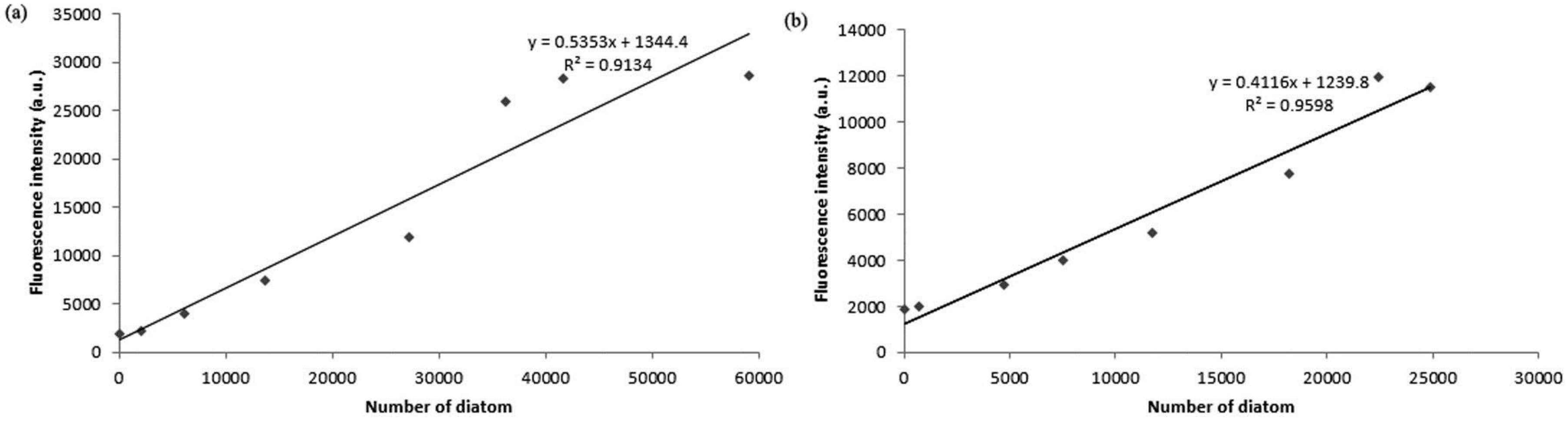

Figure S2. The relationship between fluorescence intensity and diatom density. (a) diatom from Rongcheng, China. (b) diatom from Kiel, Germany.

Table S1. Change of fluorescence intensities of attached diatoms with concentration of diatoms in suspensions after different incubation periods.

Incubation period	**Concentration of diatoms in suspension**							
	100%	**75%**	**50%**	**25%**	**12.5%**	**5%**	**0.5%**	**0**
3 h	20668	14640	8375	4258	2033	1911	1360	1373
	20019	15400	8378	3751	2271	1758	1404	1383
	21342	15253	9407	4439	2589	1809	1449	1393
	23854	16071	10672	3793	2155	2469	1384	1393
	25908	19074	9932	3242	2799	1609	1387	1379
	29522	21798	12226	3672	2432	1563	1382	1425
	26864	20774	10843	4391	1951	1531	1394	1392
	36442	18847	10802	5532	2115	2402	1417	1402
5 h	34230	23003	13528	5613	2405	1988	1406	1398
	32111	24525	15056	4562	2015	1886	1418	1402
	35927	21609	9791	5455	2426	1590	1385	1423
	29449	23304	13606	4737	2248	1947	1427	1377
	32071	21633	12015	6166	2163	1691	1411	1405
	30148	19161	11623	7559	2287	1527	1454	1426
	28194	19156	13020	3869	2306	1915	1417	1414
	31569	22563	15299	6526	2023	1794	1407	1413
6 h	25178	19856	9368	6210	1752	1956	1376	1420
	28924	22449	13425	4945	2062	1964	1417	1393
	28959	20431	12816	4611	2226	1756	1407	1405
	31764	22861	10340	4043	1903	1571	1406	1420
	28378	22283	12038	4722	1745	1486	1407	1421
	28810	23970	14779	4427	1767	2249	1412	1404
	30984	22791	12453	5259	1818	2402	1401	1394
	35224	19079	13110	4417	1780	1734	1418	1399

Table S2. Influence of season, material (ectracts vs. thalli), origin of *Gracilaria vermiculophylla* and origin of diatoms on the attachment rates of diatoms on *G. vermiculophylla*.

Source of variation	numDF	denDF	F - value	p - value
Diatom	1	468	495.980	<.0001
Origin	1	6	152.626	<.0001
Season	1	468	408.543	<.0001
Material	1	468	2070.440	<.0001
Diatom:Origin	1	468	6.405	0.0117
Diatom:Season	1	468	232.220	<.0001
Origin:Season	1	468	22.894	<.0001
Diatom:Material	1	468	539.503	<.0001
Origin:Material	1	468	80.879	<.0001
Season:Material	1	468	141.771	<.0001
Diatom:Origin:Season	1	468	5.496	0.0195
Diatom:Origin:Material	1	468	12.505	0.0004
Diatom:Season:Material	1	468	115.668	<.0001
Origin:Season:Material	1	468	28.454	<.0001
Diatom:Origin: Season:Material	1	468	12.747	0.0004

Table S3. Influence of season, material (extracts vs. thalli), origin of *Gracilaria vermiculophylla* and origin of *Ceramium* on filament attachment rates on *G. vermiculophylla*.

Source of variation	numDF	denDF	F - value	p - value
Ceramium	1	330	0.3363	0.5624
Origin	1	6	32.2522	0.0013
Season	1	330	121.3931	<.0001
Material	1	330	0.0895	0.7650
***Ceramium*:Origin**	1	330	0.1574	0.6918
***Ceramium*:Season**	1	330	8.0565	0.0048
Origin:Season	1	330	0.0084	0.9271
***Ceramium*:Material**	1	330	0.4435	0.5059
Origin:Material	1	330	12.0052	0.0006
Season:Material	1	330	3.1880	0.0751
***Ceramium*:Origin:Season**	1	330	0.0009	0.9757
***Ceramium*:Origin:Material**	1	330	3.9914	0.0466
***Ceramium*:Season:Material**	1	330	35.9222	<.0001
Origin:Season:Material	1	330	0.6730	0.4126
***Ceramium*:Origin: Season:Material**	1	330	0.1886	0.6643

Appendix S1. Transport and cultivation of *Gracilaria vermiculophylla*.

For comparing the susceptibility to pure-cultured epiphytes between native and non-native *G. vermiculophylla* populations, living *G. vermiculophylla* specimens from all sampling sites were transferred to Kiel, Rongcheng and Akkeshi. For transport, single algal individuals were wrapped in seawater-wetted tissue paper and then placed in cooling boxes while the length of transport never exceeded five days. *Gracilaria vermiculophylla* usually survived transfer under these conditions and several previous studies used this method successfully. For assays in Kiel, specimens from all sampling sites were transferred to Kiel directly, while for assays in Rongcheng and Akkeshi, individuals from all populations outside China and Japan were brought to Kiel first. Here they were kept under laboratory conditions for at least one week to allow for regeneration and were then transported to Rongcheng or Akkeshi by airplane. After their arrival, we did not observe any losses due to transportation stress and we acclimatized all specimens to laboratory conditions for at least one week prior to the assays. In Kiel, acclimation took place in indoor aquaria (14 L) which contained 500 g of *G. vermiculophylla* wet mass each. Algae were kept in seawater from the Kiel Fjord (salinity: 15 - 20) at 14 to 19 °C and a light intensity of 30 μmol m^{-2} s^{-1} for 14 h per day. Furthermore, all aquaria were permanently aerated. The water in the aquaria was completely renewed by an automatic seawater flow-through every 3.5 h (flow-through rate: 50 ml/min). Seaweeds sampled at locations with fully marine conditions were slowly acclimatized to the salinity conditions prevailing in the Kiel Fjord over the course of several days. In Rongcheng and Akkeshi, 50 g of *G. vermiculophylla* wet mass were kept in separate 1 L beakers with aeration and stored at 15 °C with 30 μmol m^{-2} s^{-1} for 14 h per day. The seawater inside the containers came from the nearby Ailian Bay (Yellow Sea water, salinity: 30 - 33) and Akkeshi Bay (Northeastern Honshu, salinity: 32), and was exchanged manually every day. Seaweeds from low salinity conditions (Baltic Sea as well as Pouldouran and Belon estuaries) were slowly adapted to the salinity conditions of Akkeshi Bay.

Appendix S2. Collection, isolation, transport and cultivation of epiphytes.

Two pennate diatoms *Stauroneis* sp. were isolated from individuals of *G. vermiculophylla* that were collected in Rongcheng and in the Kiel Fjord and transferred to 200 ml cell culture flasks (green cap, SARSTEDT®) filled with f/2 medium for pure culture. The flasks were stored in an incubator at 14 °C with 40 µmol m^{-2} s^{-1} in a 14:10 h (light/dark) photoperiod. The medium was changed every three weeks under sterile conditions.

Filaments of the red alga *Ceramium* sp. have already been used as epiphytes in assays with *G. vermiculophylla* and the two species of *Ceramium* we used for the assays commonly grow on *G. vermiculophylla* (S. Wang, pers. obs.). Individuals of *Ceramium tenerrimum* were collected in Rongcheng, while specimens of *Ceramium virgatum* stem from Kiel Fjord. Individuals of both species were collected three days before the start of the assays at sites where *G. vermiculophylla* is present. *Ceramium tenerrimum* individuals were kept under the same conditions that have been described earlier for *G. vermiculophylla* in Rongcheng, while individuals of *C. virgatum* were kept in the same way as *G. vermiculophylla* in Kiel.

Appendix S3. Establishing the relationship between diatom density and fluorescence intensity.

For this procedure, an aliquot of 100 ml of the diatom culture suspension was transferred to an Erlenmeyer flask, which was then placed on a shaker to homogenize the suspension. We then diluted it gradually 1, 1.3, 2, 4, 8, 20 and 200 times with sterile seawater that was previously filled into the wells of a 96-well plate (Microplates, BRAND plates®) to a final aliquot of 200 µl. The plate was then incubated at 14 °C for 3 h and after this time non-attached diatoms were removed by rinsing each well with 200 µl of seawater. In the following, fluorescence intensity was measured with a multitechnology plate reader (Plate chameleon, Hidex, Finland) at an excitation wavelength of 485 nm and an emission wavelength of 677 nm. After that, the 96-well plate was inspected under an inverted microscope and a photo of each well was taken. The number of diatoms on each photo was counted to identify the number of diatoms in each well and then the latter was used to identify the relationship between fluorescence intensity and diatom density (Figure S2).

Paper II

Submitted to Marine Biology

In-situ-common-garden-assays demonstrate increased chemical defence against natural fouling in non-native populations of an aquatic organism

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Abstract

The susceptibility of native and non-native populations of the red alga *Gracilaria vermiculophylla* to fouling was compared in common garden experiments. Native and non-native algae were enclosed into dialysis membrane tubes and the tubes were exposed to natural fouling. Fouling on the outside of the tubes was mediated by chemical compounds excreted by *G. vermiculophylla* that diffused through the membranes. Fouling pressure was significantly higher in the Kiel Fjord (non-native range) than in Akkeshi Bay (native range), but at both sites tubes containing non-native *G. vermiculophylla* were less fouled than those with native conspecifics. This is the first *in situ* evidence that susceptibility to fouling differs between native and non-native populations of an aquatic organism. The technique of enclosing organisms into dialysis tubes represents a simple, efficient and accurate way to test chemical antifouling defences and could possibly be applied to other organisms.

Keywords: biological invasions, chemical antifouling defence, dialysis membrane, natural fouling, *Gracilaria vermiculophylla*, non-native macroalgae

Introduction

Human-mediated introductions of marine non-native species constitute considerable threats to coastal ecosystems and related economies, such as aquaculture and fisheries, by affecting species diversity, ecosystem functions and services (Lubchenco et al. 1991; Schaffelke et al. 2006; Vitousek et al. 1996; Williams and Smith 2007). To manage such problems, it is necessary to understand the mechanisms that allow non-native species to succeed in new environments (Cacabelos et al. 2010; Johnson and Chapman 2007; Vermeij et al. 2009).

Several hypotheses have been proposed that could explain which mechanisms promote bioinvasions. One of the most widely discussed ones is the Enemy Release Hypothesis (ERH) (Keane and Crawley 2002). This concept states that non-native species have an advantage over native species as resident enemies, such as predators or parasites, cannot recognize the newly introduced resource. The Evolution of Increased Competitive Ability hypothesis (EICA) (Blossey and Nötzold 1995), an amendment of ERH, suggests that non-native species could, for the above mentioned reasons, invest more energy into growth rather than warding-off predators and parasites. This would make them more competitive than native species. Both concepts, ERH and EICA, emphasize that the successful invasions by non-native species may depend on the release from co-evolved enemies. In contrast, the Novel Weapons Hypothesis (NWH) (Callaway and Ridenour 2004) suggests that non-native species are less sensitive to enemies in the invaded habitat. This is because they possess defences which are highly efficient since the resident enemies are not adapted to them.

Most theories that seek to identify drivers of invasion success in plants or animals have been tested in terrestrial environments (Colautti et al. 2004; Keane and Crawley 2002), while less tests have been conducted in the marine realm (Wikström et al. 2006). Among the tested examples, the majority of cases are terrestrial plant-herbivore and marine seaweed-herbivore systems (Lake and Leishman 2004; Parker et al. 2006; Stastny et al. 2005; Vermeij et al. 2009). In contrast, much less is known about the effect of foulers on the invasion success of aquatic macrophytes (but see Strong et al. 2009; Svensson et al. 2013), despite the fact that epibiosis can severely impair the performance of host organisms (basibionts) (Wahl 2008). For instance, a biofilm can alter the chemical conditions at the host interface through its metabolic activities (Thevanathan 2000) and even insulate the host surface from the vital resource light (Costerton et al. 1987). Filamentous epiphytes may increase drag, decrease flexibility (Hemmi et al. 2005) and compete with the host organisms for light and nutrients (Buschmann and Gómez 1993; Honkanen and Jormalainen 2005; Wahl 1989). Some epizoans weaken the host surface and thus enhance the success of predation by crushing predators (Bach et al. 2006; Buschbaum et al. 2007). Therefore, the host organisms need to develop efficient physical or chemical defences to minimize colonization of their body surfaces. As a consequence, ecological theories such as ERH, EICA or NWH may possibly be valid for aquatic host-epibiont systems and the present study aimed at testing this *in situ* with a non-native red alga, *Gracilaria vermiculophylla* (Ohmi) Papenfuss.

Gracilaria vermiculophylla, originating from the cold and warm temperate Northwest Pacific Ocean (Tseng and Xia 1999), has invaded many coastal habitats in the northern hemisphere from the eastern Pacific (Bellorin et al. 2004; Saunders 2009) to the mid-western Atlantic (Freshwater et al. 2006; Thomsen et al. 2006) and the eastern Atlantic (Guillemin et al. 2008; Sfriso et al. 2010; Weinberger et al. 2008) during the past three decades. It has been suggested that the biotic and abiotic conditions in the recipient area may be critical for the establishment and spread of introduced marine organisms (Nyberg and Wallentinus 2009; Streftaris et al. 2005). Correspondingly, numerous studies have revealed that various intrinsic traits, such as the reproductive versatility (Abreu et al. 2011; Nettleton et al. 2013) and the ability to tolerate low light conditions, extreme temperatures (Hammann et al. 2016b), starvation (Nyberg and Wallentinus 2009), salinity (Nejrup and Pedersen 2012; Weinberger et al. 2008), ultraviolet radiation (Roleda et al. 2012) and grazers (Hammann et al. 2013) are important factors that may affect the invasion success in *G. vermiculophylla*. Moreover, previous studies show that chemical defences that protect *G. vermiculophylla* from generalist herbivores might also allow the alga to invade new habitats (Hammann et al. 2016a; Rempt et al. 2012). Further, a recent study that compared the susceptibility of native and non-native populations of *G. vermiculophylla* under identical conditions in lab bioassays demonstrated that non-native populations of this seaweed were better defended against two tested foulers than native conspecifics (Wang et al. 2016). This suggested for the first time that epibionts may compromise the performance of non-native seaweeds in their new environments to such an extent that more resistant individuals can have a selective advantage, similar as predicted by the NWH for resistance to grazing (Callaway and Ridenour 2004). However, in nature seaweeds are usually not exposed to single foulers, but to communities of various epibionts that are very diverse and highly dynamic and it is an open question whether non-native algal individuals would be also more resistant toward such assemblages than native individuals. Therefore, the present study focused on testing whether native and non-native populations of *G. vermiculophylla* differ in their susceptibility to natural fouling *in situ*.

For this study, four native *G. vermiculophylla* populations were sampled in East Asia in two adjacent ecoprovinces (following the concept suggested by Spalding et al. (2007)): The cold temperate Northwest Pacific (ecozones: Yellow Sea (China) and Northeastern Honshu (Japan)) and the warm temperate Northwest Pacific (ecozone: Central Kuroshio Current (Japan)). Furthermore, algal individuals from three non-native populations were sampled within the Northern European Seas ecoprovince (ecozones: Baltic Sea (Germany), North Sea (Germany) and Celtic Seas (France)). In addition, one non-native population from outside Europe was included, which is located in the cold temperate Northeast Pacific (ecoprovince: Oregon, Washington, Vancouver coast and shelf). The objective was to expose individuals from all populations to the same natural fouling and to compare their resistance. So far no biogeographic comparisons ever tested directly whether susceptibility to natural fouling differs among aquatic organisms originating from different ecological zones or even realms. This is because the methodological challenge is considerable: To directly expose organisms originating from different populations to the same natural fouling pressure they need to be released into the same environment, which would be unethical. To overcome this difficulty, algal specimens of different origin were in the present study individually enclosed into dialysis tubes and in this form exposed in the sea. In addition, the intensity of natural fouling pressure in native and non-native habitats was also compared.

Material and methods

Experiment 1: Monitoring of fouling pressure on *Gracilaria vermiculophylla* and on artificial substrata in native and non-native habitats

To compare the total abundance of foulers and the composition of fouling communities on *G. vermiculophylla* between native and non-native populations of the alga, exposure trials were conducted in the Kiel Fjord (54°19'48.5"N 10°8'58.8"E), Germany (non-native range of *G. vermiculophylla*) and in Ailian Bay (37°10'22.4"N 122°34'38.5"E), Rongcheng, China (native range of *G. vermiculophylla*), respectively, from May to July 2014. Each month 15 intact individuals of *G. vermiculophylla* were collected manually from the shallow subtidal in both sites. During transport from the collection sites to the respective nearby laboratory, algal individuals were kept separately in 3 l plastic bags, which were placed in cooler boxes.

Prior to the trials, all visible fouling organisms were removed from the surface of *G. vermiculophylla* with a soft brush that did not damage the host. During the cleaning, the algae remained submersed in seawater to prevent desiccation. After that, 5 g of each algal individual were put into a standardized net bag made of polypropylene with a mesh size that allowed foulers (such as juvenile invertebrates) to enter the bags (Novanet Kunststoff, maximum length: 20 cm, maximum width: 10 cm, mesh width: 9 mm). In order to assess the fouling pressure on non-living surfaces in the respective study area, 15 PVC panels (10 × 10 cm) roughened with 60 grit sandpaper were exposed at the same site as the algae and retrieved monthly during the time of the trials.

For exposure, PVC panels and net bags filled with algal individuals were paired one to one and each pair was tied to a single rope with a distance of 5 cm between plate and bag. A stone was tied to each PVC panel to stabilize it in the water column and to ensure that it remained vertically orientated. Thereafter, each pair was deployed at a depth of 0.5 m. Fouling organisms that established on the panels as well as on the living algae during the course of one month were fixed in a 4% formalin-seawater solution and their abundance and composition were then identified and quantified using a stereomicroscope. Coverage by fouling species on the panels and on *G. vermiculophylla* was assessed as percentage ranging from zero to 100%. Foulers were identified to the lowest possible taxonomic level, but due to the small size of many recruits, taxonomic resolution was often restricted to the class.

Experiment 2: Susceptibility to in situ fouling in native and non-native *Gracilaria vermiculophylla*

Individuals of *G. vermiculophylla* were collected at two occasions between June and August 2015 at eight sites located in five different countries within the native and the non-native range of the species (Table 1). All sampling areas were semi-exposed shallow bays and estuaries. Although the algal material was collected at eight different sites, the common garden field experiments were performed at only two locations: at the institute's pier of

Table 1 Geographic locations of the sampling sites and timing of sampling events for native and non-native populations of *Gracilaria vermiculophylla*.

Origin	Collection site	Geographic Coordinate
Native	Rongcheng, China, Yellow Sea	37°9'4.29"N, 122°33'35.60"E
	Qingdao, China, Yellow Sea	36°3'0.6"N, 120°20'59.1"E
	Akkeshi, Japan, Northeastern Honshu	43°1'25.80"N, 144°52'47.20"E
	Tokyo, Japan, Central Kuroshio Current	35°19'25.72"N, 139°38'8.30"E
Non-native	Kiel, Germany, Baltic Sea	54°21'9.7"N, 10°8'34.2"E
	Nordstrand, Germany, North Sea	54°29'10.0"N 8°48'44.8"E
	Pouldouran, France, Celtic Seas	48°45'57.30"N, 3°12'2.50"W
	Port Moody, Canada, Vancouver Coast	49°16'47.99"N, 122°51'6.08"W

GEOMAR, Kiel Fjord, Germany, in June and August and at the pier of the Akkeshi Marine Station, Akkeshi Bay, Japan, in July 2015 (due to technical and financial constraints, experiments 1 and 2 could not be conducted at the same location in the native range). To compare susceptibility to *in situ* fouling between native and non-native *G. vermiculophylla* populations, living algal specimens from all sampling sites were transferred both to Kiel and Akkeshi. For transport, algal individuals were individually packed in plastic bags with seawater-moistened paper tissue, and these bags were then placed in cooling containers. The length of transport, even between Kiel and Akkeshi, never exceeded 5 d. In June 2015, for equipping the field experiments in Kiel (conducted in June) and Akkeshi (conducted in July), specimens from all populations, were transferred to Kiel first. Then half of the individuals from all populations outside Japan were transported from there to Akkeshi by air cargo. After their arrival, the algae were carefully inspected, but no loss was observed due to transportation stress. Populations inside Japan were also sampled in June 2015 and the collected algal individuals were transported to Akkeshi directly. For the experiments conducted in Kiel in August 2015, which were not repeated in Japan, algal individuals from all sampling sites were transferred to the laboratory in Kiel directly. In all experiments, algal specimens were acclimatized to the locally prevailing abiotic conditions (water temperature and salinity) for at least one week prior to exposure in the field. In Kiel, this took place in a climate room with constant water temperature (15°C) and light (30 µmol m^{-2} s^{-1}) in a 12 h light-dark cycle. The algal material was kept in permanently aerated 14 l aquaria filled with seawater from the Kiel Fjord (salinity: 15–18), which was completely renewed by an automatic seawater flow-through every 3.5 h (flow-through rate: 50 ml/min). Seaweeds sampled under high salinity conditions (e.g. in Rongcheng and Qingdao) were acclimatized to the salinity conditions in Kiel Fjord over the course of several days by decreasing salinity by two units per day over a period of one week. In Akkeshi, algae were kept individually in separate beakers with aeration, which were placed in a climate room at 18°C and with light conditions of 20 µmol m^{-2} s^{-1} in a 12 h light-dark cycle. The seawater used for algal cultivation was obtained from the nearby Akkeshi Bay (salinity: 30–33) and was exchanged daily. Seaweeds from low salinity environments (Baltic Sea and Pouldouran estuary) were slowly adapted to the condition in Akkeshi Bay by increasing salinity by two units per day over a period of one week.

Algae from all populations (replication n = 5 per population), as well as a control group of five algal mimics, i.e. bundles of cut black plastic cable ties in a size similar as the algal individuals, were exposed to natural colonization by epibionts. To prevent the release of spores, fragments or microorganisms associated with *G. vermiculophylla* into the water column, algal individuals (5 g each) and algal mimics were put into dialysis membrane tubes (Spectra/por® 6 Membrance, MWCO: 1 kD) which are manufactured from natural cellulose reconstituted from cotton linters. The dialysis membrane allows molecules with a molecular weight of less than approximately 1 kD, e.g. secondary metabolites, to pass but it holds back the much larger *G. vermiculophylla* spores and microorganisms. Each of these tubes was then fixed at a water depth of 0.5 m to a vertically orientated rope, while a small stone was tied to the end of each rope to ensure vertical orientation and to stabilize it in the water column. A distance of 1 m was kept between the single ropes. Pretests had indicated that neither dialysis tubes nor *G. vermiculophylla* show signs of degradation when they are exposed under such conditions for four weeks. Exposure time during experiment 2 was 14 d in the Kiel Fjord and 18 d in Akkeshi Bay and again, no signs of algal stress like change in pigmentation or necrosis were observed. After exposure the dialysis membrane tubes were retrieved from the water and unfolded for inspection under a stereomicroscope. Settlement of fouling organisms on the lateral area was quantified by estimating percent cover for diatoms and by counting the number of individuals in case of solitary foulers. These data were obtained from three circular plastic frames (Ø 1 cm) per tube, which were placed randomly on the unfolded membranes. Furthermore, the total abundance of fouler species was estimated as percent cover within one randomly placed frame (6 × 6 cm) that was placed on each membrane.

Statistical analyses

Compositions of fouling communities (Experiment 1) were statistically compared by one-factorial Analysis of Similarity (ANOSIM) and by non-metric multi-dimensional scaling ordination (nMDS) using PRIMER 6. Prior to the analyses, data points were excluded in case the fouling abundance of a sample was zero and in case the average abundance of one fouler across all samples was smaller than 0.5%. All further statistical and graphical analyses were done using the free statistical computing software R (R Development Core Team 2014). Mixed-effect modelling was used for analyzing the total fouling coverage (Experiment 2). The modelling included two fixed factors: (1) '*Gracilaria* origin' with the levels 'Native' and 'Non-native', (2) 'Exposure site' with the levels 'Japan' and 'Germany'. Furthermore, the various sampling sites of *G. vermiculophylla* were included as a random factor. The abundances of the single fouler species were analyzed in separate approaches using one-way designs with the factor '*Gracilaria* origin' with the levels 'Native' and 'Non-native' (Experiment 2). As fouler settlement rates in Akkeshi Bay were low and, as a consequence, the resulting data set contained many zeros, it was analysed using Kruskal-Wallis rank sum tests, which do not require normally distributed data. To analyse the fouling experiments that were done in Kiel, one-way ANOVAs (in case of normal data) or Kruskal-Wallis rank sum tests (in case of non-normal data) were conducted. To achieve homogeneity of variances and normality of errors, data for *Mytilus* post-larvae and for diatoms that were obtained in June, were log-transformed prior to the one-way ANOVA, while a Welch adjusted one-way ANOVA was used for *Ulva* and for diatoms quantified in August. The t-test was used to compare fouling on dialysis tubes containing mock substrates in both exposure sites. Homogeneity of variances was checked graphically on the base of residual plots, while normality of errors was verified by histograms of the residuals and by the Shapiro–Wilk test.

Results

Experiment 1: Monitoring of fouling organisms on *Gracilaria vermiculophylla* and artificial substrata

Overall, in the Kiel Fjord six different taxonomic classes were identified both on living (*G. vermiculophylla* thalli) and non-living (PVC panels) substrata. In Rongcheng, four classes were identified on *G. vermiculophylla* individuals, while seven classes were recorded on PVC panels (Fig. 1). Also, the taxonomic groups observed on PVC panels and on *G. vermiculophylla* in the same site differed considerably (Fig. 1). Both at Kiel and Rongcheng, one of the two most abundant taxa on *G. vermiculophylla* were Ciliates. The most abundant group on *G.vermiculophylla* at Rongcheng were red algal epiphytes (Florideophyceae, primarily of the genera *Ceramium* and *Polysiphonia*), while the second most abundant group on *G. vermiculophylla* at Kiel were Bryozoans of the class Gymnolaemata. In contrast, PVC panels in Rongcheng were dominated by green algal epiphytes (primarily of the genus *Ulva*) and diatoms. These groups were also abundant on PVC panels at Kiel, but the most abundant groups on PVC panels at Kiel were bivalves of the genus *Mytilus* and barnacles of the genus *Amphibalanus* – two groups that were completely absent at Rongcheng.

Interestingly, averaged across both substrata, the total abundance of foulers was higher in Kiel than in Rongcheng (Fig. 1, 2). The total abundance of foulers was generally lower on *G. vermiculophylla* individuals than on PVC panels and this was the case at both study sites (Fig. 1, 2). In Kiel, this difference was 16% (*Gracilaria* 37 ± 25% and PVC panels 53 ±

30%, mean ± SD) and Bacillariophyta, Bivalvia and Maxillopoda were consistently less abundant on *Gracilaria* than on panels. In Rongcheng the difference was about 10 % (*Gracilaria* 8 ± 5% and PVC panels 18 ± 14%) and Ciliata and Florideophyceae were consistently more abundant, while Bryozoans of the class Gymnolaemata were consistently less abundant on *Gracilaria* than on panels.

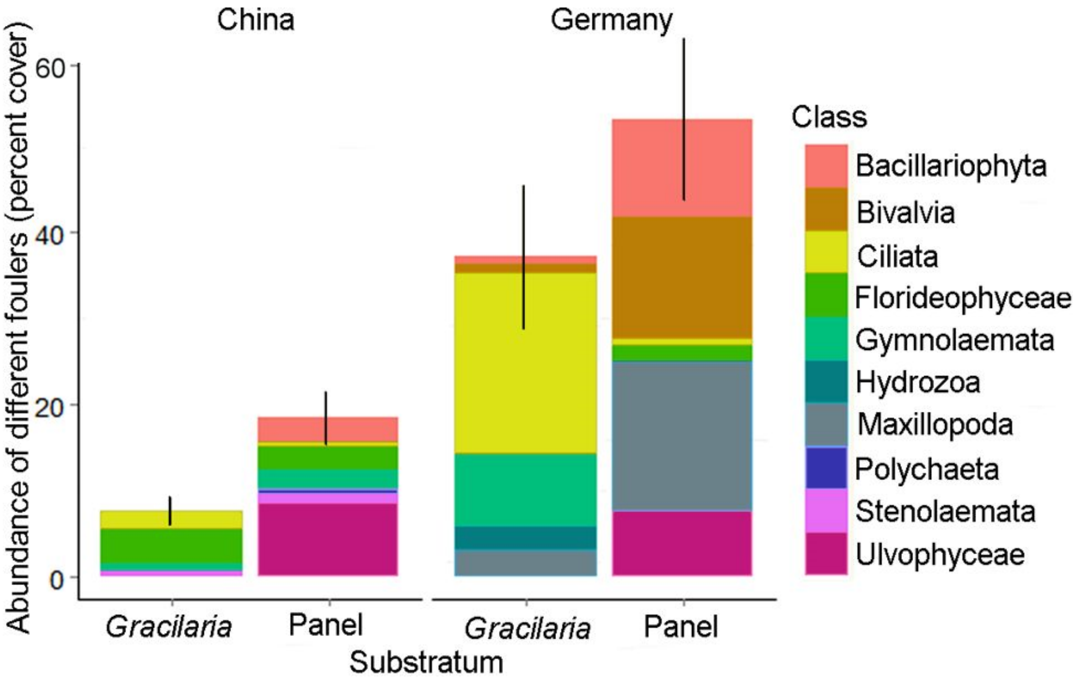

Fig. 1 Composition of fouling communities that established on *Gracilaria* individuals and on PVC panels at sites in the native (China) and non-native (Germany) range of *Gracilaria vermiculophylla* in May, June and July 2014. Means and 95% CIs (n = 45).

Across both exposure sites and all months, the compositions of fouling communities on *G. vermiculophylla* and on PVC panels (factor ‘Substratum’) were significantly different, but there was a certain overlap as indicated by ANOSIM (R=0.312, P=0.001, Fig. 3). Across both substrata and all months, the picture was the same for the factor ‘Exposure site’ (ANOSIM: R=0.538, P=0.001, Fig. 3). Across both substrata and both exposure sites, the composition of fouling communities was not very different among months (ANOSIM: R=0.239, P=0.001, Fig. 3). In Germany, however, the composition of fouling communities on *G. vermiculophylla* in May was very different from that in June and July, and the same was true for fouling communities on PVC panels in China (Fig. 3).

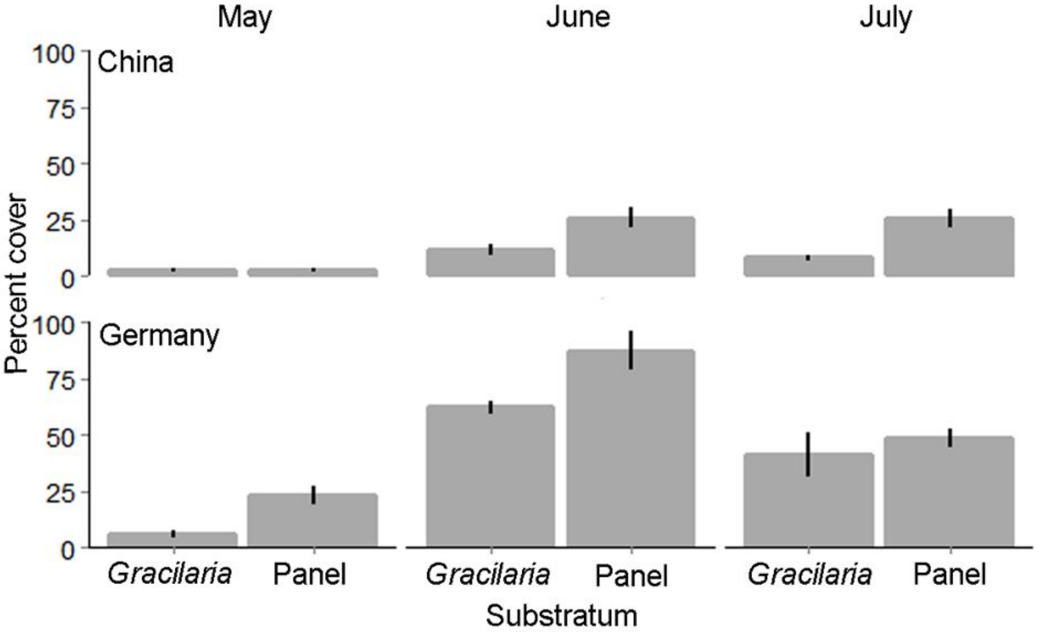

Fig. 2 Abundance of fouling communities that established on *Gracilaria* individuals and on PVC panels at sites in the native (China) and non-native (Germany) range of *Gracilaria vermiculophylla* in May, June and July 2014. Means and 95% CIs (n = 15).

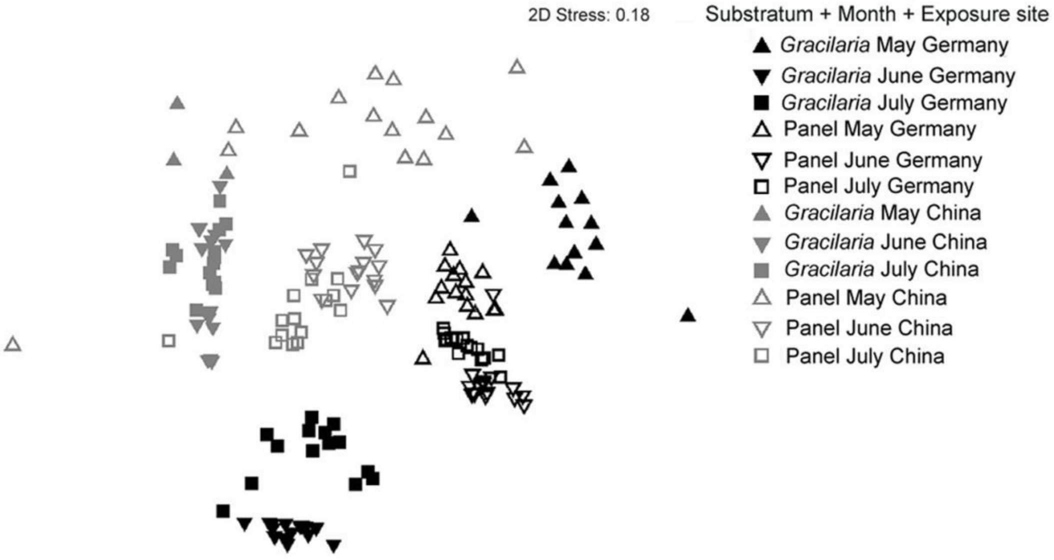

Fig. 3 Similarity between fouling communities that established on *Gracilaria* individuals and on PVC panels at sites in the native (China) and non-native (Germany) range of *Gracilaria vermiculophylla* in May, June and July 2014 (n = 15) determined by multidimensional scaling.

Experiment 2: Susceptibility to in situ fouling in native and non-native *Gracilaria vermiculophylla*

Overall, the composition of fouling communities on dialysis tubes exposed in the Kiel Fjord (Fig. 4) was more similar to that observed on PVC panels than to that observed on *G. vermiculophylla* exposed at the same site in experiment 1 (Fig. 1). It consisted of diatoms, bivalves of the genus *Mytilus*, barnacles of the genus *Amphibalanus*, green algae of the genus *Ulva* and red algae of the genus *Ceramium*. This contrasted with the site in Japan, where Bivalves, barnacles and *Ulva* were absent, but sessile Polychaetes were observed in addition to diatoms and red algae of the genus *Ceramium* (Fig. 4).

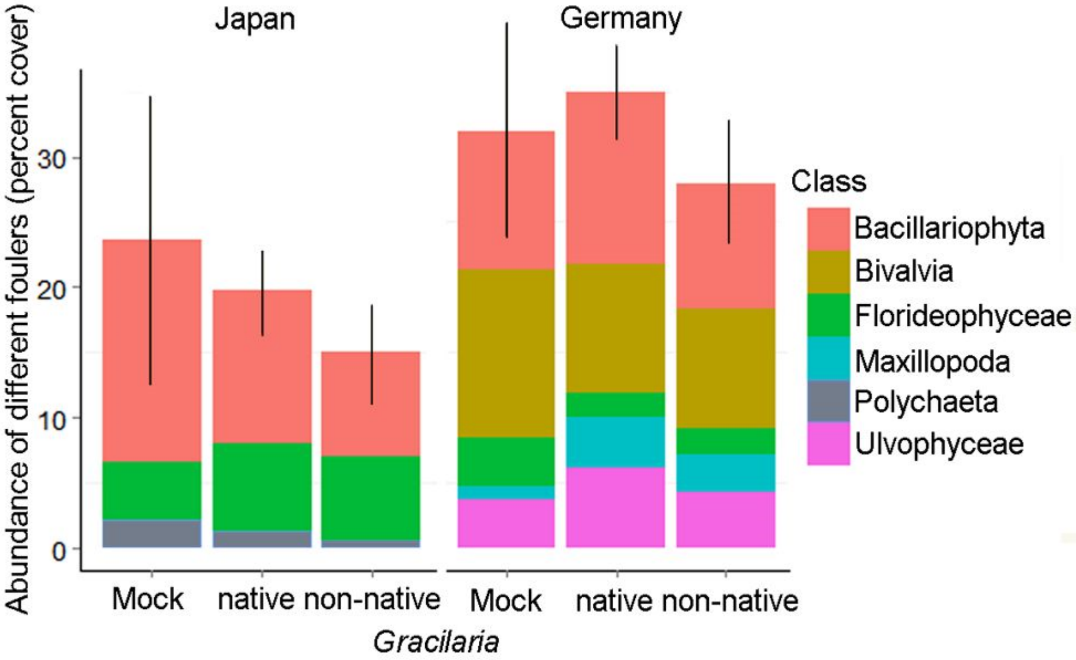

Fig. 4 Composition of fouling communities that colonized dialysis tubes filled with mock, native and non-native *Gracilaria vermiculophylla*. Tubes were exposed to natural fouling in Japan (Akkeshi Bay) and in Germany (Kiel Fjord) in June to August 2015. Means and 95% CIs (n =5 to 40).

The mean abundance of foulers on dialysis tubes containing native or non-native *Gracilaria* or control mock substrates was always higher in Germany than in Japan (Fig. 4). This difference was not statistically significant when only tubes containing mock substrates were considered (t test, P = 0.2852), which was due in part to limited numbers of replication of such tubes (n = 5 in Japan and n = 10 in Germany). However, dialysis tubes containing *G. vermiculophylla* were significantly more fouled in Germany than in Japan (Fig. 4, Table 2). Further, all fouling experiments revealed that dialysis membrane tubes that contained algal individuals from non-native populations of *G. vermiculophylla* were less fouled than those filled with algae from native populations. The size of the difference was, on average, 6% and it was statistically significant (Fig. 4, Table 2) and less than 0.001% of the unexplained variation was found to be actually covered by the random factor 'Site'. No interaction was detected between '*Gracilaria* origin' and 'Exposure site' (Table 2).

Table 2 Influence of the origin of *Gracilaria vermiculophylla* (native vs. non-native range) and the site of exposure (native/Japan vs. non-native/Germany) on natural fouling rates on dialysis membrane tubes containing living algal individuals.

Source of variation	**numDF**	**denDF**	**F - value**	**p - value**
Gracilaria origin	1	6	9.7881	0.0204
Exposure site	1	100	38.3475	<.0001
Origin : Site	1	100	0.3064	0.5811

Results from mixed effect-modelling. numDF degrees of freedom in the numerator, denDF degrees of freedom in the denominator

In June, five fouling species (*Mytilus edulis*, *Amphibalanus improvisus*, *Ulva* sp., *Ceramium tenuicorne*, diatoms) were found on the dialysis tubes that were exposed in the Kiel Fjord (Fig. 5). With the exception of *Ulva* sp. and *C. tenuicorne*, the differences in fouling rates between tubes with native and non-native *G. vermiculophylla* were statistically significant: *M. edulis*, *A. improvisus* and diatoms settled by 56%, 73% and 8%, respectively, less on dialysis tubes with non-native individuals than on those with native conspecifics (Table 3). In August, four fouling species (*A. improvisus*, *Ulva* sp., *C. tenuicorne*, diatoms) were present on the dialysis tubes in the Kiel Fjord (Fig. 6). All of them settled significantly less on tubes with non-native *G. vermiculophylla* individuals: *A. improvisus* by 59%; *Ulva* sp. by 58%; *C. tenuicorne* by 52% and diatoms by 12% (Table 4).

Table 3 Influence of the origin of *Gracilaria vermiculophylla* (native vs. non-native range) on natural fouling rates on dialysis membrane tubes filled with individuals of the alga exposed to natural fouling in Germany (Kiel Fjord) in June 2015.

Fouler	**Test**	**df**	**SS**	**MS**	**chi-squared**	**F - value**	**p – value**
Mytilus edulis	one-way ANOVA	num df = 1, den df = 103	18.37	18.368		12.21	0.0007
Amphibalanus improvisus	Kruskal-Wallis rank sum test	1			5.9353		0.01484
Ulva sp.	one-way ANOVA	num df = 1, den df = 103	242	241.8		0.898	0.346
Ceramium tenuicorne	Kruskal-Wallis rank sum test	1			2.8974		0.0887
Diatoms	one-way ANOVA	num df = 1, den df = 103	9.94	9.937		17.2	6.94e-05

df degrees of freedom, SS sums of squares, MS mean squares

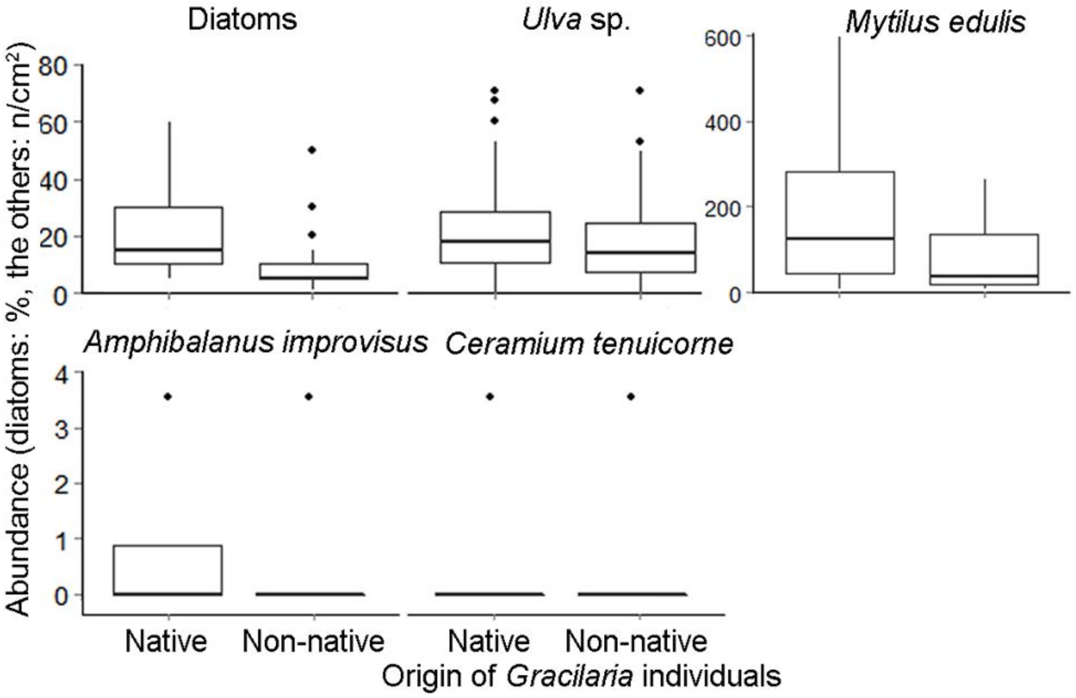

Fig. 5 Abundances of different foulers that colonized dialysis tubes filled with individuals of *Gracilaria vermiculophylla* from either native or the non-native populations of the alga. Tubes were exposed to natural fouling in Germany (Kiel Fjord) in June 2015. The abundance of diatoms was determined as % of substrate surface covered, abundance of other groups was determined by counting. Boxplots show medians, interquartiles and outliers (n = 15 to 20).

Table 4 Influence of the origin of *Gracilaria vermiculophylla* (native vs. non-native range) on natural fouling rates on dialysis membrane tubes filled with individuals of the alga exposed to natural fouling in Germany (Kiel Fjord) in August 2015.

Fouler	**Test**	**df**	**SS**	**MS**	**chi-squared**	**F - value**	**p – value**
Amphibalanus improvisus	one-way ANOVA	num df = 1, den df = 118	93.9	93.91		14.92	0.0002
Ulva sp.	Welch adjusted one-way ANOVA	num df = 1, den df = 89				43.5808	2.893e-09
Ceramium tenuicorne	Kruskal-Wallis rank sum test	1			10.6459		0.0011
Diatoms	Welch adjusted one-way ANOVA	num df = 1, den df =100				16.4181	0.0001

df degrees of freedom, SS sums of squares, MS mean squares

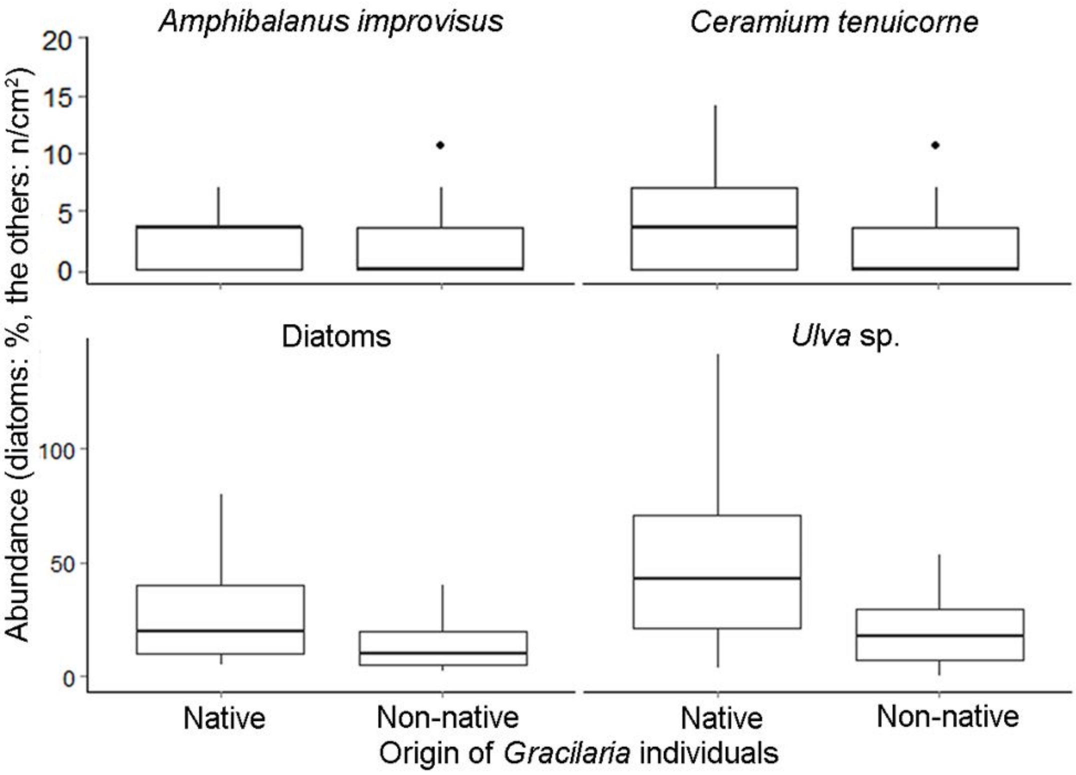

Fig. 6 Abundances of different foulers that colonized dialysis tubes filled with individuals of *Gracilaria vermiculophylla* from either native or the non-native populations of the alga. Tubes were exposed to natural fouling in Germany (Kiel Fjord) in August 2015. The abundance of diatoms was determined as % of substrate surface covered, abundance of other groups was determined by counting. Boxplots show medians, interquartiles and outliers (n = 20).

In Akkeshi, three fouling species (*Circeis spirillum*, *Ceramium kondoi*, diatoms) were recorded on the dialysis membrane tubes filled with *G. vermiculophylla* (Fig. 7). Diatoms settled significantly less (by 3%) on tubes containing non-native *G. vermiculophylla*, while the differences for *C. spirillum* (50% less on non-native *G. vermiculophylla*) and *C. kondoi* (18% less on non-native *G. vermiculophylla*) were marginally significant (Table 5).

Table 5 Influence of the origin of *Gracilaria vermiculophylla* (native vs. non-native range) on natural fouling rates on dialysis membrane tubes filled with individuals of the alga exposed to natural fouling in Japan (Akkeshi Bay) in July 2015.

Fouler	df	chi-squared	p - value
Circeis spirillum	1	3.4495	0.0633
Ceramium kondoi	1	3.573	0.0587
Diatoms	1	9.963	0.0016

Results from Kruskal-Wallis rank sum tests. df degrees of freedom

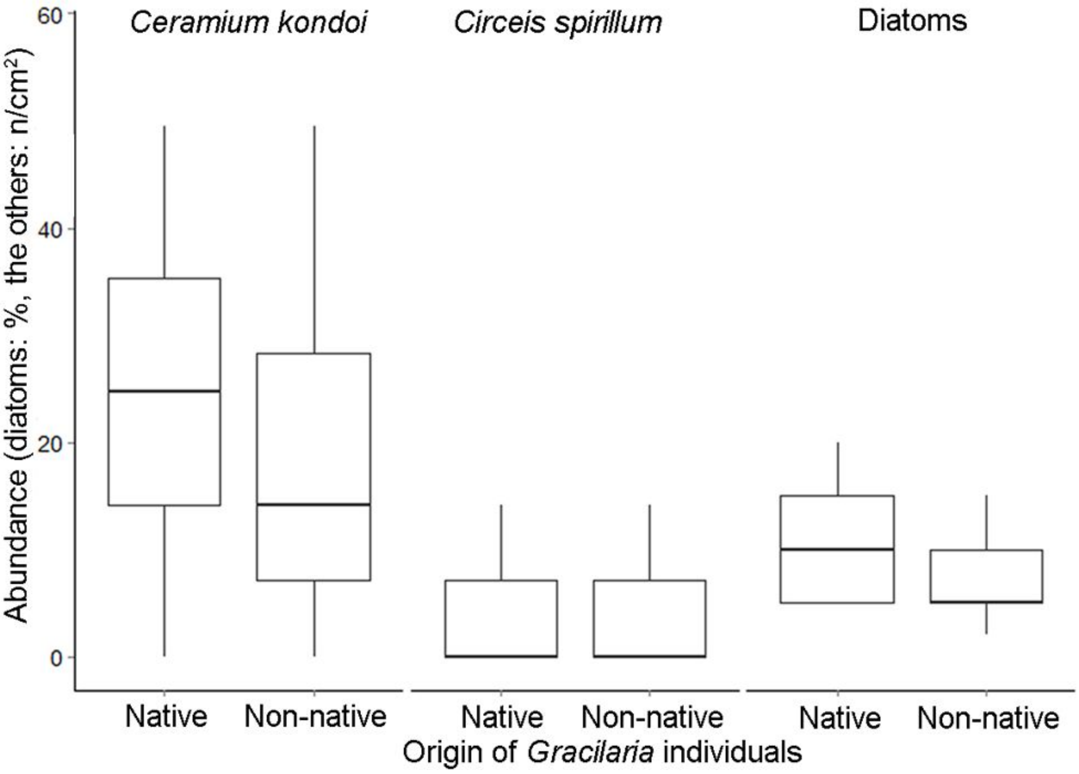

Fig. 7 Abundances of different foulers that colonized dialysis tubes filled with individuals of *Gracilaria vermiculophylla* from either native or the non-native populations of the alga. Tubes were exposed to natural fouling in Japan (Akkeshi Bay) in July 2015. The abundance of diatoms was determined as % of substrate surface covered, abundance of other groups was determined by counting. Boxplots show medians, interquartiles and outliers (n = 15 to 20).

Discussion

Differences in fouling pressure between locations in *Gracilaria*'s native and non-native range

The monitoring of field fouling pressure revealed that both substrata – living *G. vermiculophylla* individuals and PVC panels – were more heavily fouled in the Kiel Fjord, Germany (non-native range of *G. vermiculophylla*) than in Ailian Bay, Rongcheng, China (native range). Also in the field fouling experiments with dialysis tubes containing *G. vermiculophylla*, less fouling was observed in the native range (Akkeshi Bay, Japan) than in the non-native range (Kiel Fjord, Germany). Thus, both sets of experiments provided similar results, although the native study sites were located in different ecozones. This suggests that, at least during summer, *G. vermiculophylla* faces a more severe fouling pressure in its new environment in the Kiel Fjord.

There are various possible explanations for the higher fouling pressure in the Kiel Fjord. First of all, it could be that the system there is generally more productive than the two Asian sites, due to higher nutrient concentrations in the eutrophic environment of the Western Baltic. A higher primary and secondary productivity would also mean that fouling organisms and their propagules occur in higher abundances and fouling rates should therefore be higher in eutrophic than in oligotrophic systems (Korpinen et al. 2007). However, nutrient concentration data available for the Kiel Fjord, Ailian Bay and Akkeshi Bay do not support the assumption that eutrophication is generally higher in the Kiel Fjord (suppl. Table S1). Much rather, the fact that the Kiel Fjord is a semi-enclosed environment without significant wave action and tides, while Rongcheng Bay and Akkeshi Bay are sea areas with heavy wave action and pronounced tidal amplitudes and turbulent stress could explain the difference in fouling rates. Crimaldi et al. (2002) showed that turbulent stress events influence larval settlement success and Koehl et al. (2013) found that wave action induced by ship wakes can reduce settlement rates by fouling organisms. It should be noted that the monitorings conducted to quantify the fouling pressure only considered one location in the non-native range of *G. vermiculophylla*. Therefore it cannot be said for sure whether sites in the non-native range are generally subject to more fouling pressure than sites in the native range of *G. vermiculophylla*. In any case the finding in this study certainly contradicts and therefore falsifies the predictions of the ERH and EICA (Blossey and Nötzold 1995; Keane and Crawley 2002), which both suggest that introduced species should experience a reduction in the diversity and the abundance of co-evolved enemies in their new range compared to their native range. Indeed, most marine fouling organisms are relatively unspecific with respect to host choice (Wahl and Mark 1999) and in this light the probability of release from specialized fouling organisms during invasions of aquatic organisms appears as relatively low.

***Gracilaria*’s defence against fouling organisms is, at least partly, based on secondary metabolites**

A second observation while monitoring the fouling pressure in the field was that foulers at both testing sites were generally more abundant on PVC panels than on *G. vermiculophylla* individuals and only few groups of foulers (e.g. Ciliates) were more abundant on the alga. Although there are many differences, such as shape, between the two substrata, this finding suggests the presence of a physical and/or chemical antifouling defence in *G. vermiculophylla* against most foulers. A similar picture was reported by Rickert et al. (2015), who observed higher densities of the barnacle *A. improvisus* on PVC panels compared to the thalli of two nearby *Fucus* species. In an experimental approach, the authors could identify surface-bound metabolites as the reason for the deterrent effect that *Fucus* showed against settlement of *A. improvisus*. Evidence of chemical defences against algal macrofoulers has also been reported for *Gracilaria chilensis*, a species that is phylogenetically relatively closely related with *G. vermiculophylla* (Lion et al. 2006). Moreover, previous studies revealed that extractable surface-bound metabolites from *G. vermiculophylla* mediate the defences of *G. vermiculophylla* against epibacteria (Saha et al. 2016), diatoms and *Ceramium* filaments (Wang et al. 2016). In contrast, physical antifouling defence strategies, such as epithallus sloughing and gelatinous or microstructured surfaces – that have been reported from some macroalgae (da Gama et al. 2014; Yamamoto et al. 2013) – were so far not observed in *G. vermiculophylla* by us or others. Nonetheless, given that absence of evidence is no evidence of absence it cannot be excluded with certainty that physical defence mechanisms contributed to the difference in fouling rates that was observed between *G. vermiculophylla* thalli and non-living surfaces. However, the inclusion of *G. vermiculophylla* into dialysis tubes in experiment 2 not only prevented algal spores or associated microorganisms from entering the water column, but it also excluded any impact of physical antifouling defence mechanisms of *G. vermiculophylla* on foulers. The approach thus permits to evaluate the extent to which algal excretion of metabolites affects fouling organisms. Interestingly, the fouling communities on dialysis tubes containing *G. vermiculophylla* were more similar to fouling communities on PVC panels than to fouling communities that developed directly on the surface of the algae, and this could suggest that relevant physical defence mechanisms against foulers exist in *G. vermiculophylla*. However, algal specimens that originated from different populations affected the fouling communities on dialysis tubes in different ways. This not only indicates that chemical defences exist, but it also strongly suggests that the capacity for such defences varies among populations. Using dialysis membranes that contained living algae instead of artificial substrata coated with surface extracts as in our previous study (Wang et al. 2016) allowed us to assess the capacity of *G. vermiculophylla* for chemical antifouling defences in a more accurate way. This is because extracted metabolites are usually subject to oxidation and other degradation processes, while living algae warrant for a relatively constant release of undegraded compounds. No signs that the algae suffered from being kept in tubes, such as a change in colour or necrosis, were observed during the field fouling experiment.

Defence strength rather than avoidance by colonizers was responsible for the observed inter-population differences in fouling rates

The fouling experiments with living *G. vermiculophylla* thalli enclosed in dialysis membranes revealed that individuals from non-native populations of the alga were generally less susceptible to fouling than native conspecifics. This was the case in both test areas. Therefore, since non-native algal individuals were exposed to natural fouling in both the native and the non-native range of *G. vermiculophylla*, it could be excluded that a lower preference of native colonizers for the non-native macroalga was the exclusive reason for the observed picture. Thus, the observed picture certainly resulted from differences in the composition and/or concentration of bioactive metabolites that were released from the surface of *G. vermiculophylla* and leached through the dialysis membranes. This result is consistent with findings of a previous study (Wang et al. 2016), in which epiphytes originating from both distribution ranges of *G. vermiculophylla* exhibited less readiness to settle on surface extracts of non-native specimens than on surface extracts of native individuals.

However, it cannot be explained with certainty why the antifouling defence capacity of *G. vermiculophylla* changed during the invasion process. The simplest explanation is that fouling resistant genotypes were selected during the invasion process, i.e. during transport and establishment in the new habitat, and that their frequency is therefore higher in non-native than in native populations. This scenario would be in agreement with theories that predict a selection of an increased defensive capacity during biological invasions, such as the NWH. In its non-native distribution range *G. vermiculophylla* is mostly found in extremely sheltered lagoons and estuaries (Weinberger et al. 2008). In contrast, populations in the native range – including Qingdao and Rongcheng - are often located on more wave exposed rocky shores. As outlined above wave exposure generally reduces fouling pressure and in this light the selection of an increased antifouling defence may have facilitated the settlement and spread of *G. vermiculophylla* in non-native environments with particularly high fouling pressure, such as the Kiel Fjord.

Alternatively, the increased antifouling capacity of non-native *G. vermiculophylla* could result from an adaptation to other biotic pressures, such as grazing. Non-native *G. vermiculophylla* populations are more strongly defended against herbivores than native populations (Hammann et al. 2013) and compounds that deter consumers are sometimes also active against epibionts. For example, in *Asparagopsis armata* Bromoform was shown to deter not only mesograzers, but also microsettlers (Paul et al. 2006a; Paul et al. 2006b). Likewise, the defence of *G. vermiculophylla* against herbivores is at least partially due to a capacity for production of oxylipins from arachidonic acid after wounding (Hammann et al. 2016a) and those compounds were shown to also deter algal epibionts of Gracilarioids (Lion et al. 2006). Non-native populations of *G. vermiculophylla* were already shown to have a stronger capacity for production of oxylipins than native populations (Hammann et al. 2016a). However, oxylipin production requires activation by heavy wounding (Rempt et al. 2012), and although certain foulers of Gracilarioids (i.e. *Ceramium* species) can cause tissue wounding when they anchor themselves in the host (Leonardi et al. 2006) such wounding was excluded in this experiment: Direct, immediate contact of host and foulers was prevented by the dialysis tubes. Therefore, other compounds than oxylipins must be responsible for the deterrent effects observed in this study. Nontheless, the increased capacity for production of those unidentified defence compounds in non-native populations of *G. vermiculophylla* could also provide additional defence strength against other biological enemies that are not foulers.

A third explanation could be that a release from other and more severe biotic pressures (e.g. grazing) in the new environment of the alga led to a re-allocation of resources into antifouling defence (Blossey and Nötzold 1995). In Akkeshi, Japan, local populations of *G. vermiculophylla* were heavily grazed by the amphipod species *Caprella scaura* and *Ampithoe lacertosa* during summer 2015 and in Rongcheng, China, another *Caprella* species was also intensively feeding on *G. vermiculophylla* during summer and autumn 2014 (S. Wang, pers. obs.). In contrast to this, in Kiel, Germany, the overall grazing pressure on *G. vermiculophylla* appears as generally lower than in Asia (Hammann et al. 2013). If this reduced feeding pressure generally applies to non-native habitats then *G. vermiculophylla* in these habitats could possibly allocate more resources to defences against generalist foulers than native populations (Blossey and Nötzold 1995).

Furthermore, it is possible that the intense fouling pressure in the non-native habitat stimulated the antifouling defence in *G. vermiculophylla.* Such a demand-driven antifouling defence regulation has been suggested by Saha and Wahl (2013) and Rickert et al (2016). Saha and Wahl (2013) found that the anti-settlement activity of *F. vesiculosus* at two geographically distinct locations in Germany (Gelting and Poel) varied temporally, reaching a peak in summer/autumn, which was in phase with the density of bacterial cells in the plankton. Rickert et al (2016) showed that in individuals of the two *Fucus* species *F.*

vesiculosus and *F. serratus*, which inhabit adjacent habitats in the Western Baltic Sea, the strength of a chemical defence against microfouling varied seasonally and that fluctuations in the defence level tend to match with fluctuations in microfouling pressure. However, such demand-driven defences against fouling in *G. vermiculophylla* would require a highly sensitive sensing system, as direct contact between host and foulers was excluded in the experiments by the dialysis tubes.

The influence of scattering over a larger geographical scale in sampling sites on the within-range variability in antifouling defence

For the intra-specific comparison presented here individuals of *G. vermiculophylla* were sampled from two different ecological realms within the non-native range of the species. However, antifouling defence primarily differed between ranges, while within-range variability of antifouling defence was low: Less than 0.001% of the unexplained variation went back to the random factor ‘site’. Thus, non-native populations in Europe and at Port Moody (E Pacific) showed a similar antifouling defence capacity. This suggests that the observed picture could generally apply to non-native populations of *G. vermiculophylla*. More comparative studies considering non-native populations of *G. vermiculophylla* in other parts of the world are needed to confirm this assumption.

In conclusion, the fouling experiments with living algae described here give the first *in situ* evidence that individuals of *G. vermiculophylla* from non-native populations are generally less susceptible to natural fouling than native conspecifics. This is true regardless of whether the non-native algal individuals are exposed in the native or in the non-native range of *G. vermiculophylla*. This indicates that the observed difference goes back to the defence properties of the algae and not to the fact that the foulers present were not able to recognize the algae as a suitable settlement substratum. Since all surface properties of the alga were excluded by enclosing them in membrane tubes, it is clear that the antifouling properties were mediated by chemical compounds which were released by the algae and which were able to pass through the membrane. However, so far there is no information about which chemical compounds are involved in this. Finally, this is the first study in which living algae enclosed in dialysis membrane tubes were exposed in the field to assess natural fouling rates. This technique represents a more simple, efficient and accurate way to test chemical antifouling defences in seaweeds than using artificial substrata coated with extracts.

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Supplementary information

suppl. Table S1. Mean nutrient concentrations in Kiel Fjord (Germany, non-native range of *Gracilaria vermiculophylla*), Ailian Bay (China, native range) and Akkeshi (Japan, native range).

Location	**Nitrate + Nitrite (µmol l^{-1}, mean ± SE)**	**Ammonium (µmol l^{-1}, mean ± SE)**	**Silicate (µmol l^{-1}, mean ± SE)**	**Phosphate (µmol l^{-1}, mean ± SE)**
Kiel Fjord	0.4 ± 0.2	1.4 ± 0.3	13.6 ± 2.4	0.6 ± 0.2
Ailian Bay	3.1 ± 0.6	0.003 ± 0.0004	2.6 ± 0.3	0.3 ± 0.04
Akkeshi Bay	0.2 ± 0.02		12.1 ± 1.5	0.9 ± 0.06

Data for Kiel Fjord are taken from Rickert et al. (2015) and were recorded from April to September 2013, nutrient concentrations in Ailian Bay were measured from May to September 2014 (Xunshan Group Co., Ltd, unpublished data), while data for Akkeshi Bay were recorded from July to August 2015 (Hokkaido National Fisheries Research Institute, unpublished data).

Paper III

Submitted to Biofouling

Seasonal variation in antifouling activity of the red macroalga *Gracilaria vermiculophylla* from the Baltic Sea

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Abstract

Marine macroalgae are constantly exposed to colonization by other sessile life forms, while the intensity of fouling pressure and the identity of colonizing organisms fluctuate spatially and seasonally. We therefore hypothesized that macroalgae adjust their antifouling defences to fouling pressure. To test this assumption fouling pressure in the Baltic Sea and the defence capacity of *G. vermiculophylla* surface extracts against two foulers - the diatom *Stauroneis constricta* and the filamentous alga *Ceramium tenuicorne* - were assessed over one vegetation period on a monthly basis. Both hexane and DCM surface extracts inhibited *C. tenuicorne* similarly, while only hexane surface extracts deterred *S. constricta*. The activities of both surface extracts exhibited significant seasonality. However, only the fluctuations in deterrence strength of DCM extracts towards *C. tenuicorne* correlated with the intensity of epiphytism by *C tenuicorne* on *G. vermiculophylla.* Thus, *G. vermiculophylla* appears to adjust some of its antifouling defences to fouling pressure.

Keywords: *Ceramium tenuicorne*, antifouling defence, fouling pressure, *Gracilaria vermiculophylla*, seasonal variation, *Stauroneis*

Introduction

Epibiosis is the spatial association between an ‘epibiont’ (the fouling organism) and a ‘basibiont’ (the fouled host organism), which is typical and ubiquitous in marine environments (Taylor and Wilson 2003, Wahl 1989, Wahl and Mark 1999). Seaweeds are photosynthetic organisms and particularly prone to colonization by epibionts, which comprise many different taxa from all kingdoms, as they are sessile and restricted to the shallow, euphotic zone where the abundance and diversity of fouling organisms is known to be high (da Gama et al. 2008, de Nys et al. 1995).

Although epibionts have not only detrimental, but also neutral or even beneficial effects on the basibiont seaweeds (Costerton et al. 1987, Thevanathan 2000), they are generally considered harmful for the performance of the host macroalga (Duffy and Hay 2000, Wahl 2008). These effects can be direct or indirect. A direct influence is given when epibionts reduce growth and photosynthesis of the host alga by competing for light and nutrients (Buschmann and Gómez 1993, Cebrian et al. 1999, Honkanen and Jormalainen 2005), when they impair the host by mechanically penetrating into the tissues (Leonardi et al. 2006), or when they cause mortality by increasing drag and weight (Hemmi et al. 2005). Indirect effects emerge when palatable epibionts raise the attractiveness of the host alga for consumers (Wahl et al. 1997).

Such negative effects on macroalgal fitness should be an important driver that leads to the selection of genotypes that possess efficient physical or chemical antifouling (AF) defence that minimizes colonization on their surfaces (Clare 1996, da Gama et al. 2014, Nylund et al. 2008, Paul and Ritson-Williams 2008). Periodical epithallus sloughing is arguably the best investigated physical defence against epibionts (da Gama et al. 2014) and has been reported for numerous species of macroalgae, such as the brown alga *Sargassum* spp. (Yamamoto et al. 2013), the red alga *Dilsea carnosa* (Nylund and Pavia 2005) or the green alga *Ulva intestinalis* (McArthur and Moss 1977). Another common physical AF mechanism among brown, red and green macroalgae is the formation of gelatinous layers that cover surfaces and hinder adhesion to them (Chapman et al. 2014, Davis et al. 2003). In addition, metabolites have been discovered in macroalgae that chemically defend them against colonizers. Most of the studies that so far focused on the last type of defence studied crude extracts which were gained from the whole tissues of algal thalli (Amade and Lemée 1998, de Nys et al. 1995, Wikström and Pavia 2004). However, to affect fouling metabolites must either be present on the surface of the host or must be released into the surrounding water at ecologically relevant natural concentrations (Nylund et al. 2007). As a consequence, some studies concentrated on testing the AF activity of surface-associated metabolites of seaweeds at ecologically relevant concentrations (Dworjanyn et al. 2006, Sudatti et al. 2008). For example, it has been reported that surface extracts of the red alga *Delisea pulchra* and the green alga *Caulerpa filiformis* at natural concentrations inhibit settlement of *Polysiphonia* sp. spores and *Ulva australis* gametes (Nylund et al. 2007). Studies with the brown alga *Fucus vesiculosus* identified relatively polar and non-polar compounds extracted from the algal surface that have the potential to impede epibiotic biofilms at natural concentrations (Saha et al. 2011, 2012). Rickert et al. (2015, 2016) also showed that surface-extracted metabolites from the brown algae *F. vesiculosus* and *Fucus serratus* deterred settlement of micro- and macrofoulers at two-fold natural concentrations.

Temperate macroalgae are almost permanently exposed to fouling pressure, which can spatially and seasonally fluctuate with regard to both the intensity and the composition of the colonizer pool (Arrontes 1990, Wahl et al. 2010). However, if the production of chemical defence is actually costly in terms of metabolic energy (Dworjanyn et al. 2006), algal defence strength should vary with factors that affect energy resources, such as temperature and light (Lehvo et al. 2001, Rickert et al. 2015) or with defence demand, such as fouling pressure (Rickert et al. 2015, Schauer et al. 2003). In accordance with this, previous studies of temperate macroalgae already documented a seasonal variation in AF activity (Hellio et al. 2004, Maréchal et al. 2004, Saha and Wahl 2013, Stirk et al. 2007).

Originating from East Asia, the perennial red alga *G. vermiculophylla* (Ohmi) Papenfuss has invaded many temperate coastal habitats in the Northern hemisphere, where temperature, light and fouling pressure undergo strong seasonal variations (Abreu et al. 2011, Hammann et al. 2013a, Sfriso et al. 2012, Weinberger et al. 2008). The alga was first discovered in the German Baltic Sea in 2005 (Schories and Selig 2006), where it can grow on soft bottom substrates as well as on stones in the shallow subtidal (Weinberger et al. 2008). The later are also an important habitat for *F. vesiculosus*, the most common native perennial alga in the Baltic Sea. Thus, in the SW Baltic both species directly compete for resources (Weinberger et al. 2008). Moreover, compared to *F. vesiculosus*, *G. vermiculophylla* experiences a lower grazing pressure in this sea area and is a preferred refuge for mesograzers and other invertebrates (Hammann et al. 2013b, Weinberger et al. 2008). A previous study on this alga has shown that surface extracts from both native and non-native populations of *G. vermiculophylla* could chemically inhibit settlement of diatoms and of *Ceramium* species

(Wang et al. 2016). Futher, oxylipins produced by a related alga, *Gracilaria chilensis*, were found to reduce colonization by algal epiphytes and can be upregulated after cell damage due to epiphyte penetration (Lion et al. 2006, Weinberger 2007). Lion et al. (2006) showed that high amounts of 8R-hydroxy eicosatetraenoic acid (8-HETE) and 7,8-dihydroxy eicosatetraenoic acid (7,8-di-HETE) generated by *G. chilensis* after wounding inhibit the settlement of spores of the red alga *Acrochaetium* sp., as well as the attachment of vegetative thalli of the epiphytic red alga *Ceramium rubrum*. However, the surface-associated compounds that are responsible for the observed AF activity of *G. vermiculophylla* so far remain unknown. The purpose of the present study was to investigate whether there is seasonal variation in this activity and whether its strength changes with the prevailing fouling pressure. This study intended to answer several questions: 1. is the fouling pressure in the field fluctuant seasonally? 2. whether the chemical AF defence in *G. vermiculophylla* varies seasonally, and 3. is the strength of chemical AF defence in *G. vermiculophylla* associated with prevailing fouling pressure?

Material and Methods

Sampling site and collection of *Gracilaria vermiculophylla*

Individuals of *Gracilaria vermiculophylla* (Ohmi) Papenfuss were collected monthly from April to October 2015 in the shallow subtidal at Tirpitzmole, Kiel Fjord, Germany (54°21'9.7"N, 10°8'34.2"E). As *G. vermiculophylla* was often found growing tangled and unattached in this area (Wang, pers. obs.), algal samples were collected at five different spots (at a distance of 20 m from each other) to avoid the sampling of clones. Immediately after collection, the seaweed material was transported in a cooler box to the laboratory, where it was gently cleaned in sterile seawater to remove associated fouling organisms and debris.

Collection and cultivation of foulers for bioassays

The pennate diatom *Stauroneis constricta* was isolated from *G. vermiculophylla* individuals and used as a test fouler in fouling bioassays. It was cultivated in an incubator at 14 °C, at a light intensity of 20 µmol m^{-2} s^{-1} in a 14:10 h light:dark cycle. The f/2 medium (Guillard and Ryther 1962) prepared with Baltic Sea water (salinity: 15 ± 2) was used for culture and changed every three weeks. The diatom suspension was applied in bioassays with a concentration of 1500-1600 cells/µl.

Filaments of *Ceramium* sp. have previously been described as epiphytes on *Gracilaria* species (Leonardi, Miravalles, Faugeron, Flores, Beltrán and Correa 2006, Lion, Wiesemeier, Weinberger, Beltran, Flores, Faugeron, Correa and Pohnert 2006, Michetti et al. 2016) and the *Ceramium* species used as a test fouler in fouling bioassays are commonly found growing on *G. vermiculophylla* thalli in the Kiel Fjord (Wang et al. 2016). Three days before fouling bioassays, specimens of *Ceramium tenuicorne* were collected from the Kiel Fjord at sites where *G. vermiculophylla* was also present. They were maintained in Baltic Sea water (salinity: 15 ± 2) that was daily exchanged, at a temperature of 16 °C with a light intensity of 20 μmol m^{-2} s^{-1} in a 14:10 h light:dark photoperiod.

Field monitoring of fouling organisms

A monitoring of the epibionts present on *G. vermiculophylla* was conducted at the institute pier of GEOMAR (54°19'48.5"N 10°08'58.8"E) in the Kiel Fjord from April to October 2015. Each month five freshly collected algal individuals were exposed to natural colonization for one month, in order to assess the total degree of fouling and the composition of established fouling communities at the respective time of the year. Before the exposure, all visible fouling organisms were removed from the surface of *G. vermiculophylla* with a soft brush to avoid any damage. After that, single algal individuals (5 g each) were placed into standardized net bags of polypropylene with a mesh size that allowed mesograzers to enter and exit the bags freely (Novanet Kunststoff, Fritzlar, maximum length: 20 cm, maximum width: 10 cm, mesh width: 9 mm). In order to investigate the actual fouling pressure during each month at the study site, five PVC panels (10 cm x 10 cm) roughened with 60 grit sandpaper were exposed as reference. Each pair of PVC panel and net bag filled with an algal individual was tied to a separate rope without overlap. Thereafter, the combination was deployed at 0.5 m below mean sea surface level with a stone, in order to remain vertically orientated. After one month of exposure, the fouling organisms that established on both sides of the panels and on living algae were fixed in a 4% formalin-seawater solution and their abundance and composition were then quantified under a stereomicroscope (10-fold magnification).

Surface extraction of *Gracilaria vermiculophylla*

Surface extractions of 80 g of *G. vermiculophylla* from each of the five sampling spots were conducted monthly, in order to collect any compounds with anti-fouling activity. Prior to extraction, ten fragments of *G. vermiculophylla* were scanned and weighed to determine the relationship between algal surface area and algal wet mass by using the imaging software Image J (National Institute of Health, Bethesda, Maryland, USA) for surface area quantification. The average surface area of 1 g algal material across all ten fragments was identified as 46.06 cm^2 g^{-1}. The total surface area of any given wet mass of *G. vermiculophylla* was therefore calculated by multiplying the wet mass (g) by 46.06 cm^2 g^{-1}.

Before extraction, the algae were spin-dried in a centrifuge (Eppendorf 5810 R, Eppendorf, Hamburg, Germany) at 15 °C and with 200 rpm for 30 s. Surface extraction was then carried out as described in detail in Wang et al. (2016). Briefly, *G. vermiculophylla* individuals were dipped for 5 s into 500 ml of a constantly stirred mix of dichloromethane (DCM) and hexane 1:4 (v/v), a treatment that causes no damage of epidermal cells (Wang et al. 2016). The resulting solution was filtered through a paper filter (Ø 185 mm, Macherey-Nagel, Düren, Germany) into a 1000 ml Duran flask to remove particles and then evaporated under vacuum at 30 °C with a rotary evaporator. The residue was re-dissolved sequentially in pure hexane and DCM, respectively, to obtain a non-polar and a polar fraction. Both fractions were finally dissolved in 4 ml of the respective solvent and stored in 4 ml vials at -20 °C. Solvent controls were also prepared by simply evaporating the same amount of solvents as for the surface extraction, and re-dissolving the residue in pure hexane and DCM as described above.

Fouling bioassays with *Stauroneis constricta*

Laboratory fouling bioassays with *S. constricta* that compared the AF activity of all surface extracts of *G. vermiculophylla* that were collected during the preceding seven months were conducted in October 2015. Prior to these bioassays a linear relationship between diatom density and diatom fluorescence intensity at an excitation wavelength of 485 nm and an emission wavelength of 677 nm was established by measuring the fluorescence intensity of diatoms attached in 96-well plates (flat bottom, Greiner bio-one, Frickenhausen, Germany) at known density with a plate reader (Chameleon IV, Hidex, Finland). Diatom densities were quantified by counting numbers of diatoms within eight randomly taken visible fields per well under an inverted microscope (10-fold magnification).

All surface extracts were then tested at fivefold natural surface concentration in 96-well plates (flat bottom, Greiner bio-one) as described in detail in Wang et al. (2016). Briefly, extract stock solution containing extract from an algal surface that was five times as large as the surface of a well containing 100 µl was transferred to a well and diluted with pure solvent (either hexane or DCM) to a final volume of 100 µl. Control wells were loaded with pure solvent. All solvent was then evaporated *in vacuo* overnight and 100 µl of stirred diatom suspension were transferred into each well. Wells impregnated with only extracts and sterile seawater were prepared to check the background fluorescence. After inoculation the wells were incubated for 3 h under the same conditions as the diatom culture. Unattached diatoms were removed by rinsing each well with 200 µl of sterile seawater and the fluorescence intensity per well was finally recorded at an excitation wavelength of 485 nm and an emission wavelength of 677 nm. Numbers of settled diatoms per well could then be calculated based upon the linear relationship between diatom density and fluorescence intensity.

Fouling bioassays with *Ceramium tenuicorne*

Also laboratory fouling bioassays with the red alga *C. tenuicorne* were conducted in October 2015, in order to compare the AF activity of the surface extracts of *G. vermiculophylla* that were collected during the preceding seven months. These bioassays were carried out as described in detail in Wang et al. (2016). Briefly, paper filters (Carl Roth, Karlsruhe, Germany, Ø 35 mm, area: 9.6 cm²) were impregnated with extract obtained from 48 cm² *G. vermiculophylla* surface to obtain a fivefold natural surface concentrations and placed into 6-well plates. Control wells received filters impregnated with control solvent residues only. Each well was then filled with 5 ml of Provasoli's enriched seawater (PES) (Bold and Wynne 1978) and ten *Ceramium* filaments (1 cm in length) were transferred to each well. The wells were incubated at the same temperature and light conditions that had been applied during the maintenance of *C. tenuicorne* and the proportion of attached *Ceramium* filaments was quantified after two weeks.

Statistical analyses

For data from both field monitoring and bioassays with *S. constricta* and *C. tenuicorne*, the free computing software R (R Development Core Team 2014) was used for statistical and graphical analyses. The t test was used to analyze differences in the fouling abundance on field-exposed *G. vermiculophylla* individuals and on PVC panels. To analyze the bioassays with *S. constricta* and *C. tenuicorne*, t-test was employed to test differences in the mean settlement events on hexane and DCM surface extracts of *G. vermiculophylla* collected in different months of the year. The t-test was also used to test differences in the mean settlement events on surface extracts and on solvent controls. Test assumptions were checked graphically by using residual plots.

In order to test the hypothesis that seasonal AF defences oscillate annually between a peak season and a minimum season, a second order polynomial regression model was applied to detect seasonality in AF defence strengths against *S. constricta* and *C. tenuicorne*, since this study only covered a part of the year. The statistical analyses were performed in R.

A crossed Pearson correlation analysis was conducted with the software NCSS 2007 (NCSS.LLC, Kaysville, Utah) to identify time shifts in the correlations between bioassay results on one hand and field fouling pressure or actual fouling on *G. vermiculophylla*.

Results

Seasonal patterns in *in situ* fouling

The overall abundance of foulers was lower on *G. vermiculophylla* individuals than on PVC panels (Figure 1), but the difference was not statistically significant (t-test, p = 0.09).

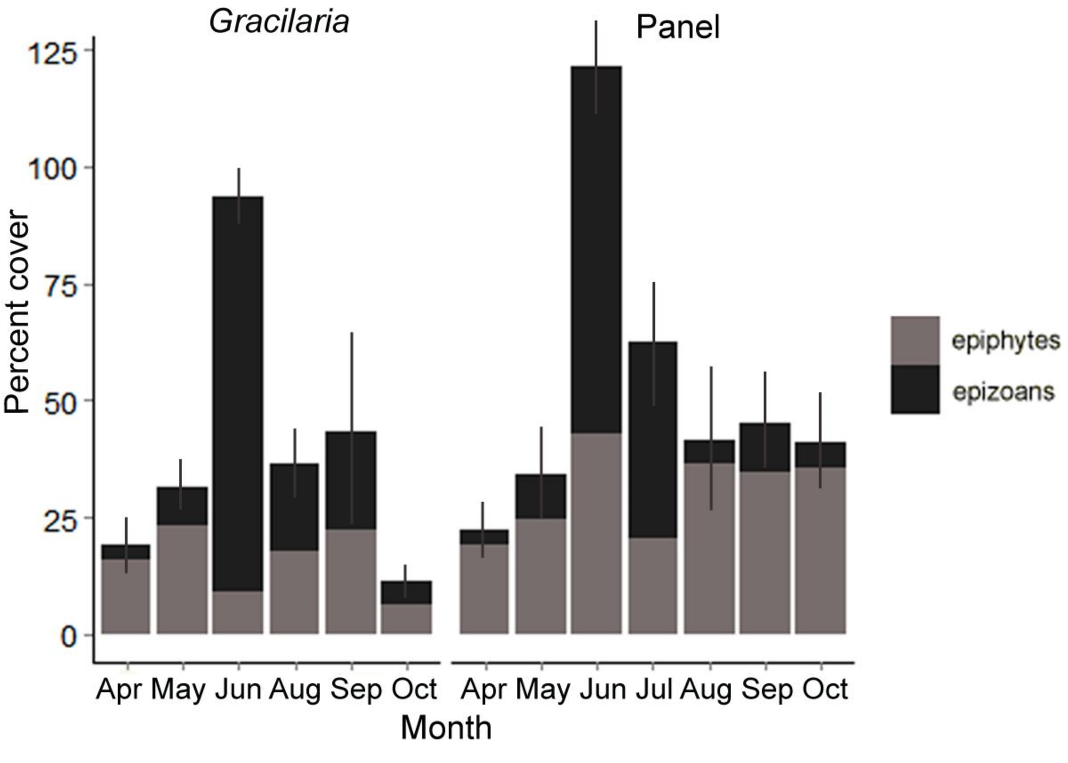

Figure 1. Seasonal variability in the mean coverage (%; ± 95% CI; n = 5) of epiphytes and epizoans recorded on *Gracilaria vermiculophylla* individuals and PVC panels in the Kiel Fjord over 7 months of one year. No data for fouling colonization on *G. vemiculophylla* individuals in July: *G. vemiculophylla* individuals were largely lost due to grazing.

Table 1. Mean coverage (%; ± SD; n = 5) of foulers on PVC panels exposed in the Kiel Fjord, Germany from April to October, 2015. Empty fields indicate absence of the respective foulers.

Species	**April**	**May**	**June**	**July**	**August**	**September**	**October**
Ceramium tenuicorne	0.1±0.22	1.42±0.95	4.31±2.02	3.7±2.41	1.67±0.58	2.5±0.5	1±0.35
Diatoms	18.1±7.77	18.1±11.18	30.9±5.59	6.5±1.37	8.33±1.44	20.5±4.47	34±8.77
Ectocarpus siliculosus						5.8±2.56	
Pilayella sp.	0.2±0.27	3.1±0.74		0.7±1.04	20±9.01		
Porphyra sp.							0.2±0.27
Punctaria tenuissima		0.2±0.27				0.8±1.79	
Ulva sp.	0.81±0.66	1.9±0.96	6.1±2.19	9.6±3.07	6.33±3.33	5.2±2.97	0.3±0.27
Alcyonidium gelatinosum				1±0.35	0.17±0.29	1.1±0.65	0.2±0.27
Amphibalanus improvisus		1.9±0.82	12.9±6.14	22.5±7.07	4.33±1.44	5.4±1.64	0.9±0.22
Conopeum serati				1.5±1.37		0.2±0.45	
Electra pilosa		2.3±1.6					
Focculina	0.9±0.22	1.8±1.52	0.5±0	0.4±0.22		0.5±0	0.5±0.35
Forraminifera	0.3±0.45	0.1±0.22					0.1±0.22
Halitholus yoldia-arcticae			0.1±0.22				
Laomedea sp.						0.5±0.61	2.7±1.89
Musculus marmoratus		3.5±2.47		0.1±0.22			
Mytilus edulis	2±0.5		65±11.73	15±6.37	0.33±0.58	2.5±1	0.7±0.67
Nudibrachia	0.1±0.22						
Polydora sp.				0.4±0.65	0.17±0.29	0.2±0.45	
Aurelia polyps			0.2±0.27	1.2±0.91			
Ostracoda							0.6±0.22
Cordpylophora caspia						0.1±0.22	

Specifically, the overall abundance of epiphytes on PVC panels was almost twice as much as on *G. vermiculophylla* individuals (t-test, $p < 0.0001$), while there were similar amounts of animal foulers on both substrata (t-test, $p = 0.93$).

On the PVC panels, the most intense epiphytic fouling occurred in June (41.31 ± 4.41%, mean coverage ± SD) (Figure 1) and overall diatoms, *Ceramium tenuicorne* and *Ulva* sp. were the most common epiphytic foulers. Diatoms showed a maximum in coverage in October, *C. tenuicorne* in June, and *Ulva* sp. in July and August (Table 1). Epifaunal coverage of the panels was lower than cover by epiphytes from April to May and from August to October, but not in June (78.7 ± 11.22%) and July (42.1 ± 10.57%) (Figure 1). In these months *Amphibalanus improvisus* and *Mytilus edulis* were the most common epizoans and they exhibited a maximum coverage in June and July and in June, respectively (Table 1).

On *G. vermiculophylla* individuals, epiphyte fouling peaked in May (23.4 ± 4.28%) (Figure 1). Diatoms and *C. tenuicorne* were the most common epiphytes here with maximum covers in May and August, respectively (Table 2). In contrast with the PVC panels *Ulva* sp. was found on *G. vermiculophylla* only in June and only at very low abundances (Table 2). Similar to what observed on the PVC panels, epifaunal coverage was lower than epiphyte cover in all months, except of June and August (Figure 1). It exhibited a distinct peak in June (84.4 ± 5.59%), due to the substantial occurrence of *M. edulis* during this month (Table 2).

Table 2. Mean coverage (%; ± SD; n = 5) of foulers on *G. vermiculophylla* individuals exposed in the Kiel Fjord, Germany from April to October, 2015. No data could be recorded in July because *G. vemiculophylla* individuals had disappeared due to grazing. Empty fields indicate absence of the respective foulers.

Species	April	May	June	August	September	October
Aglaothamnion sp.				1±1	1.4±0.55	
Ceramium tenuicorne		1±0	3.4±2.19	10±0	6±2.24	1.8±0.45
Diatoms	16±5.48	22±4.47	5±0	6.67±2.89	14±5.48	4.4±1.34
Monostroma grevillei			0.4±0.55		0.4±0.55	
Polysiphonia sp.					0.4±0.89	
Porphyra sp.		0.4±0.55				0.2±0.45
Ulva sp.			0.2±0.45			
Amphibalanus improvisus		0.2±0.45	0.2±0.45	1.33±0.58	0.4±0.55	0.2±0.45
Clava multicornis		0.6±0.55				
Corophium sp.		0.4±0.55				
Focculina		1.4±0.55				
Laomedea sp.	0.2±0.45	2.4±2.41	0.2±0.45	0.33±0.58	4.2±3.56	2.8±1.48
Mytilus edulis	2.6±1.34	3±1.22	84±5.48	16.67±5.77	16±19.17	1.6±0.55
Polydora sp.				0.33±0.58	0.6±0.55	0.4±0.55
Aurelia polyps		0.2±0.45				
Graveia fransicana	0.2±0.45					
Alcyonidium gelatinosum	0.2±0.45					

Seasonality in the AF activity of surface extracts against *Stauroneis constricta*

Averaged across all months fewer diatoms (by 13%) attached to surfaces covered with non-polar compounds (extracted with hexane) than to those coated with polar compounds (extracted with DCM) and this difference was statistically significant (t-test, p = 0.006, Figure 2). However, also averaged across all months neither Hexane-based nor DCM-based surface extracts modified the settlement of diatoms compared to solvent controls (t-test, hexane: p = 0.7, DCM: p = 0.33, Figure 2). Nonetheless, hexane surface extracts of *G. vermiculophylla* exhibited a seasonality in their AF activity against *S. constricta*. Adaptation of a polynomial regression function detected a minimum in the settlement of diatoms on surfaces coated with these extracts between July and August (p < 0.0001, Figure 3a) and the lowest mean diatom settlement was observed in August (Figure 3a). The activities of DCM surface extracts against diatoms, however, did not show a seasonal pattern (p = 0.8, Figure 3b).

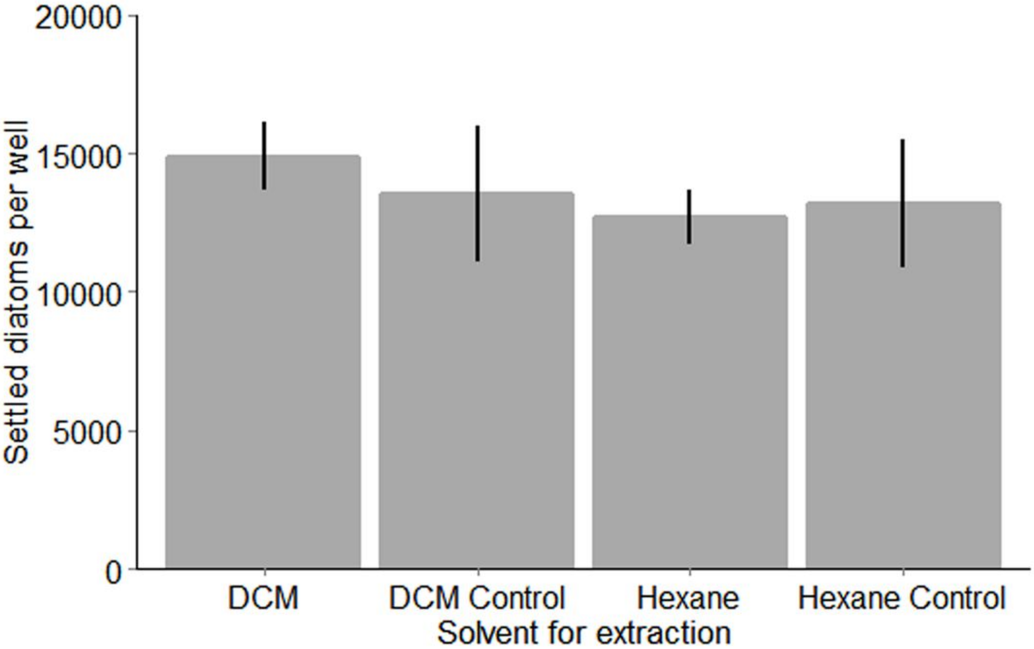

Figure 2. Mean numbers of diatoms settled on *G. vermiculophylla* surface extracts obtained with Hexane and DCM. Error bars: 95% CI, n = 280 in each solvent extract group and n = 32 in each solvent control group.

Although a seasonal pattern in the AF effect of hexane extracts was detected, crossed Pearson correlation analysis did not find evidence that diatom deterrence by hexane-based surface extracts was correlated with the intensity of diatom fouling pressure (= diatom settlement density on PVC panels) during or preceding the sampling for extract preparation (Table 3). Moreover, monthly variations in the AF activity of hexane extracts were also not correlated with diatom settlement density on *G. vermiculophylla* before, during or after the sampling time (Table 3). Significant correlations were also not detected between diatom settlement on PVC panels or *G. vermiculophylla* and the deterrent effect on diatoms of DCM-based extracts (Table 3).

Table 3. Relationships of field fouling pressure by diatoms and *Ceramium tenuicorne* and actual fouling of these organisms on the surface of *Gracilaria vermiculophylla* with the AF defense strength of surface extracts from this seaweed. Results from a crossed Pearson correlation analysis. Settlement of diatoms on either PVC panels or *G. vermiculophylla* in different months was cross-correlated with AF defense strength of hexane and DCM surface extracts against the diatom *S. constricta*. In the same way settlement of *C. tenuicorne* on both substrata was cross-correlated with defense strengths against this epiphyte.
A time shift of 0 indicates that settlement on the substratum and defense strength were recorded at the same time. Negative time shifts indicate that settlement on the substratum was recorded prior to the defense strength. Positive time shifts indicate that the defense strength was recorded first. They were not of interest and therefore not tested in the case of PVC panels, since an effect of algal antifouling defenses on the subsequent settlement on nonliving surfaces makes ecologically no sense. Bold numbers indicate correlations with p < 0.05, italics indicate correlations with p < 0.06.

Substratum	**Time shift [months]**	**Bioassays with hexane- and DCM-based surface extracts and foulers**							
		Hexane:Diatom		DCM:Diatom		Hexane:*Ceramium*		DCM:*Ceramium*	
		r	p	r	p	r	p	r	p
PVC panels	-2	-0.5850	0.3001	0.7085	0.1805	-0.1417	0.8202	0.0032	0.9960
	-1	0.5078	0.3038	0.2486	0.6347	-0.0672	0.8993	*-0.7974*	*0.0574*
	0	0.3820	0.3978	-0.6515	0.1129	0.0421	0.9286	-0.6656	0.1027
Gracilaria	-2	0.1574	0.8426	-0.1095	0.8905	0.7665	0.2335	0.7098	0.2902
	-1	0.4371	0.4618	-0.3772	0.5314	0.8173	0.0911	0.3259	0.5925
	0	0.4875	0.3267	-0.1980	0.7069	-0.1496	0.7772	*-0.7982*	*0.0570*
	1	0.0693	0.9118	0.4768	0.4168	-0.1155	0.8533	**-0.9177**	**0.0280**
	2	0.0945	0.9055	0.2039	0.7961	0.7950	0.2050	-0.1156	0.8844

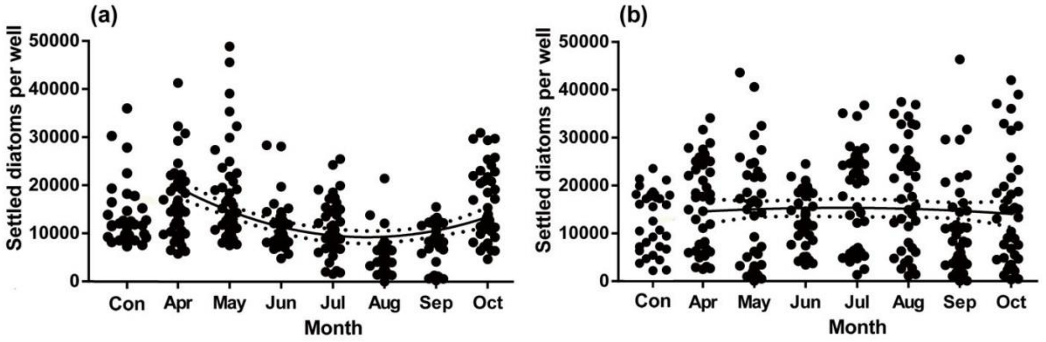

Figure 3. Mean numbers of diatoms settled on *G. vermiculophylla* surface extracts obtained with (a) Hexane and (b) DCM in different months (n = 40 in each month). Solvent controls are indicated as "Con" (n = 32 in each case). Lines represent best fitting second order polynomial regression function, dotted lines indicate 95% CI.

Seasonality in the AF activity of surface extracts against *Ceramium tenuicorne*

Averaged across all seasons *Ceramium* filaments attached marginally less (by 1%) to surfaces coated with DCM surface extracts than to such covered with hexane surface extracts, but this small difference was not statistically significant (t-test, $p = 0.44$, Figure 4). Both surface extracts significantly repelled settlement of *Ceramium* filaments relative to the respective control across all monthly samples (t-test, hexane: $p = 0.002$, DCM: $p = 0.002$, Figure 4). Similar to findings in the bioassays with diatoms, the AF activity of hexane surface extracts against *C. tenuicorne* showed a seasonal pattern. Adaptation of a polynomial regression function detected the lowest *Ceramium* filament attachment between June and July ($p < 0.0001$, Figure 5a), although the lowest monthly mean of *Ceramium* attachment was recorded in August (Figure 5a). A significant seasonal pattern was also found in bioassays conducted with DCM surface extracts ($p = 0.003$, Figure 5b). Adaptation of a polynomial regression function detected the lowest number of *Ceramium* filaments attached on surfaces coated with DCM extracts that were gained between July and August, and the lowest monthly mean of *Ceramium* attachment was recorded in July.

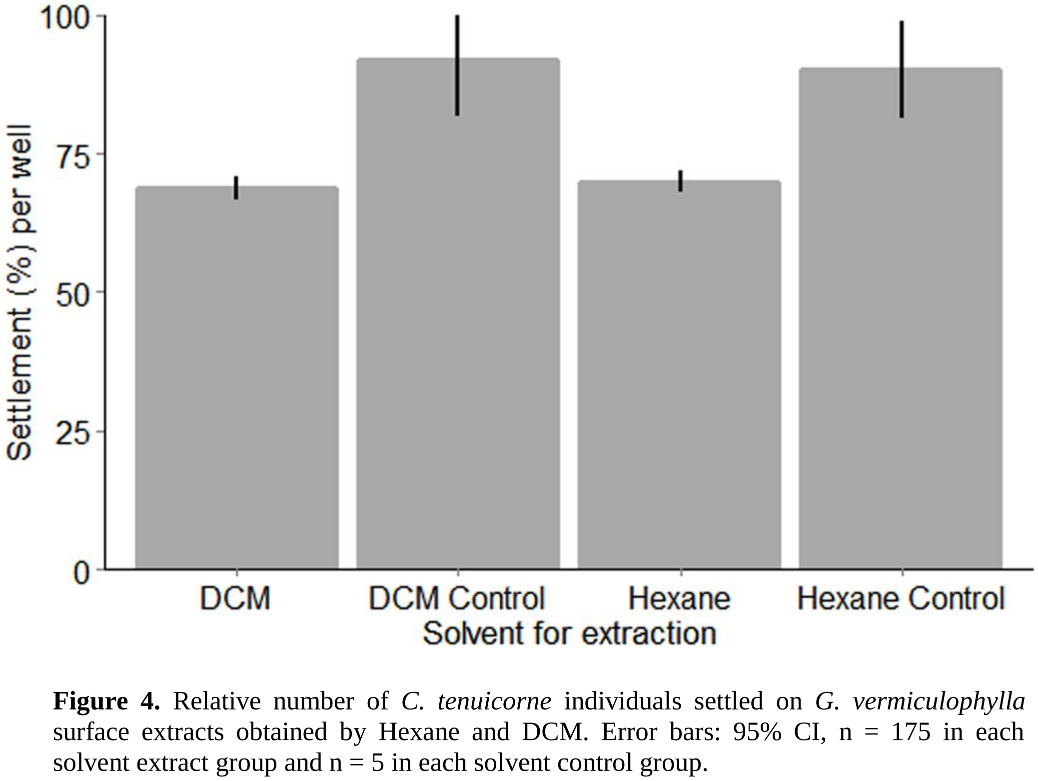

Figure 4. Relative number of *C. tenuicorne* individuals settled on *G. vermiculophylla* surface extracts obtained by Hexane and DCM. Error bars: 95% CI, n = 175 in each solvent extract group and n = 5 in each solvent control group.

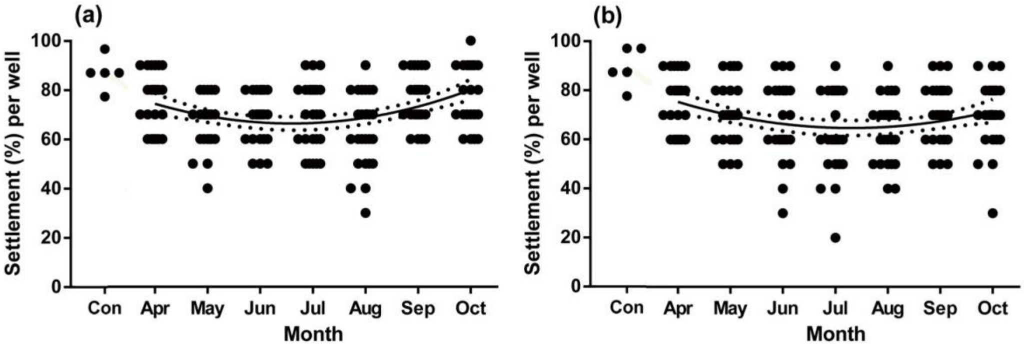

Figure 5. Relative number of *C. tenuicorne* individuals settled on *G. vermiculophylla* surface extracts obtained by (a) Hexane and (b) DCM in different months (n = 25 in each month). Solvent controls are indicated as "Con" (n = 5 in each case). Lines represent best fitting second order polynomial regression function, dotted lines indicate 95% CI.

Crossed Pearson correlation found no evidence that monthly variations in the deterrent effects of hexane-based surface extracts towards *C. tenuicorne* were correlated with the fouling pressure (as settlement on PVC panels) by *C. tenuicorne* before or at the sampling time (Table 3). However, a weakly significant negative correlation was detected between the settlement density on DCM surface extracts and the settlement density on PVC panels one month before the extraction. Thus, surface extracts tended to deter *C. tenuicorne* strongly when the extraction followed a month with generally high fouling pressure by *C. tenuicorne* (Table 3, Figure 6a). Further, the settlement density on DCM surface extracts exhibited a significant negative correlation with the settlement density of *C. tenuicorne* on *G. vermiculophylla* one month after the extraction (Table 3, Figure 6b). In other words, the settlement density of *C. tenuicorne* on *G. vermiculophylla* became particularly low when the sampling followed a month with high deterrence towards *C. tenuicorne*. A weakly significant negative correlation was also detected between the settlement density on *G. vermiculophylla* extracts and the settlement density on *G. vermiculophylla* at the extraction time (Table 3, Figure 6c). In contrast, the deterrent effect of hexane extracts towards *C. tenuicorne* did not correlate with the settlement density of this epiphyte on *G. vermiculophylla* (Table 3).

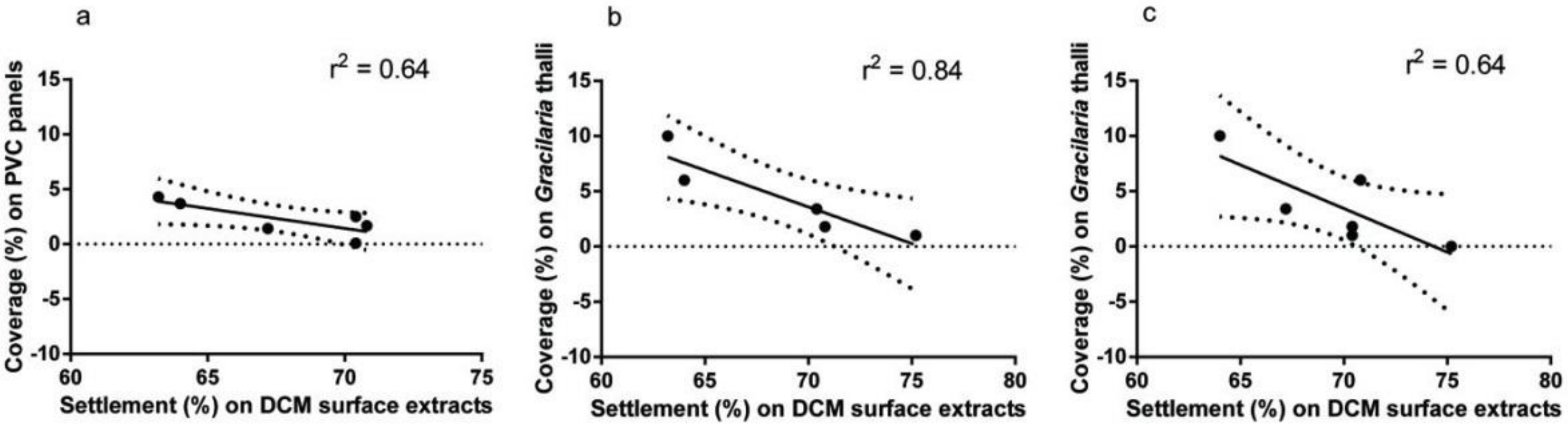

Figure 6. Linear regressions of the mean relative numbers of *C. tenuicorne* settled on DCM surface extracts from *G. vermiculophylla* (n = 25 per month) in different months with the mean density (in all cases = 5 per month) of *C. tenuicorne* (a) on PVC panels in the preceding month, (b) on *G. vermiculophylla* thalli in the following month and (c) on *G. vermiculophylla* thalli in the same month. Lines represent regression functions, dotted lines represent 95% CI.

Discussion

Gracilaria vermiculophylla is less susceptible to fouling than non-living substrates

In the field monitoring fouling organisms were detected across all months, and their abundance was consistently lower on *G. vermiculophylla* individuals than on PVC panels. Apparently *G. vermiculophylla* has a capacity to deter or suppress parts of the settlers. Many previous studies have also shown that fouling organisms preferred artificial over living substrata and suggested that this difference was due to host defences (Brock et al. 2007, Dobretsov and Wahl 2001, Rickert et al. 2015). For instance, in all given microhabitats larvae of the blue mussel *Mytilus edulis* significantly preferred artificial over living algal substrata (Dobretsov and Wahl 2001). Likewise, Rickert et al. (2016) observed higher densities of diatoms settled on a reference substrate than on two rockweeds, and suggested that host surface metabolites and host cuticle shedding could make the algal surface unattractive to diatoms. Given that extractable surface-associated metabolites from non-native specimens of *G. vermiculophylla* generally have a stronger capacity to deter foulers than those from native individuals (Wang et al. 2016) and also given that the present study was conducted with non-native specimens, *a priori* a high level of chemical defence capacity against fouling should be expected. Mechanical traits such as surface shedding have so far not been observed in *G. vermiculophylla*, which is not a proof that they do not exist.

Interestingly, the reduced capacity to settle on *G. vermiculophylla* compared to PVC panels was only statistically significant when epiphytes were regarded. This seemingly contrasts with numerous studies that demonstrated the existence of efficient chemical or physical defences against animal foulers in seaweeds. For example, surface-associated metabolites from *Fucus* deter the settlement of barnacles (Rickert et al. 2015) and Bryozoans are shed off by the red alga *Dilsea carnosa* (Nylund and Pavia 2005). However, also in the present study the barnacle *Amphibalanus improvisus* and the Bryozoan *Electra pilosa* exhibited in all months under investigation higher mean densities on PVC panels than on *G. vermiculophylla*, while the very abundant blue mussel *Mytilus edulis* and Hydrozoans of the genus *Laomedea* showed an opposite trend (Tables 1 and 2). In this light it gets obvious that animal foulers are not generally insensitive to the AF defence of *G. vermiculophylla*. Much rather, those animal foulers that are only locally attached to the algal surface (such as *Laomedea* and *Mytilus*) are apparently more capable to tolerate the algal defence than those that attach firmly to larger connected areas of the thallus surface (such as *Amphibalanus* and *Electra*).

Fouling on *Gracilaria vermiculophylla* is subject to seasonal variation and correlates with antifouling defence

As expected, the abundance of both epiphytes and epizoans consistently fluctuated on PVC panels and algal thalli seasonally and it peaked in summer. Given that important factors for the reproduction and/or growth of fouling organisms also shift seasonally, such dynamics are usually observed (Lehvo et al. 2001). Fouling pressure increased from spring toward summer when water temperature and light availability went up and decreased again toward autumn. In the preceding year a similar study had been conducted with PVC substrates in the same Bay and it also recorded maximal fouling pressure in summer, although the summer peak was less pronounced for animal settlers than in the present monitoring (Rickert et al. 2015). This confirms again the important impact of matching or mismatching climatic, physical and biological key conditions on the development of fouling communities (Patel et al. 2003. Prendergast 2010).

Similar as in a previous study (Wang et al. 2016), only non-polar compounds (extracted with hexane) from *G. vermiculophylla* surfaces had a deterring activity against *S. constricta* in bioassays, and this deterrence was only observed during parts of the year. In contrast, hexane and DCM surface extracts inhibited *C. tenuicorne* similarly. Apparently the deterrent activities of compounds associated with *G. vermiculophylla* surfaces are species-specific, i.e. the compounds which mediate defences against *S. constricta* and *C. tenuicorne* are different and have different polarities. This also gets apparent when seasonal patterns in the activities of these extracts were compared: the AF activity of hexane extracts peaked between July and August towards *S. constricta*, but already between June and July towards *C. tenuicorne*, suggesting that both organisms were not affected by the same compounds within these extracts. Further, the activity of DCM extracts towards *C. tenuicorne* did not peak in between June and July, but between July and August, which indicates that *G. vermiculophylla* employs at least two different compounds that both target this epiphyte, and undergo divergent seasonal cycles. Similar observations of multiple deterrents in one organism that target the same foulers are not rarely observed (Saha et al. 2011, 2012). For example, Saha et al. (2011, 2012) demonstrated that surface-associated polar and non-polar metabolites from *Fucus vesiculosus*, i.e. dimethylsulphopropionate (DMSP), proline and fucoxanthin all inhibited the attachment of the same bacterial strains. Given that our surface extracts were obtained with only two solvents (i.e. hexane and DCM) that cover a limited part of the polarity spectrum, additional deterrent compounds may be present in *G. vermiculophylla* that could not be extracted, e.g. water-soluble compounds.

We hypothesized that the chemical AF capacity could be adjusted to seasonally shifting fouling pressure. However, our observations only provide limited evidence of such a relationship. In the case of both foulers, the deterrent effects of Hexane-based or DCM-based extracts were not significantly correlated with the fouling pressure (measured as fouler settlement on PVC panels) before or at the sampling time. However, a relatively high *in situ* abundance of *C. tenuicorne* tended to precede a relatively high deterrence strength of DCM surface extracts towards this fouler by one month with $p < 0.058$. Thus, a higher fouling pressure exerted by *C. tenuicorne* - but not by diatoms - may modulate an increasing AF defence in the host that reaches its maximum after one month. This finding is somehow consistent with our expectation that *G. vermiculophylla* should defend itself against fouling more efficiently in phases with intense fouling pressure. A correlation study by Rickert et al. (2016) similarly observed that a high *in situ* prokaryotic fouling pressure preceded a low prokaryotic settlement on *Fucus serratus* surface extracts by one month. Further, a relatively high deterrence strength of DCM surface extracts towards *C. tenuicorne* preceded a relatively low settlement density of this fouler on *G. vermiculophylla* by one month. Additionally, a weakly significant correlation was also detected between the deterrence strength of DCM extracts and the settlement density of *C. tenuicorne* on *G. vermiculophylla* at the extraction time. This confirms that the defensive capacity of DCM-soluble compounds on the surface of *G. vermiculophylla* is of ecological relevance, since it apparently influences *C. tenuicorne* settlement not only in bioassays or on impregnated non-living surfaces, but also on the host alga.

However, we found no correlational evidence that the mean level of surface colonization by *C. tenuicorne* on *G. vermiculophylla* affects the subsequent mean level of AF defences of *G. vermiculophylla* towards the same fouler, possibly because various other factors that were not controlled may also influence epibiosis. For example, *C. tenuicorne* probably interacted not only with *G. vermiculophylla*, but also with competing foulers and grazers, which may have weakened the correlation between its presence and host defences. Similarly, the absence of correlations between deterrence of surface extracts and diatom settlement on both PVC panels and on *G. vermiculophylla* thalli could be due to the circumstance that *S. constricta* was the only diatom species used in the bioassays, while *in situ* fouling pressure involves numerous taxa of diatoms.

The data for this study were collected during only one single seasonal cycle and it cannot be fully excluded that a different outcome might have been observed during another year with different meteorological and hydrographic dynamics. Nonetheless, we may conclude that fouling pressure in the SW Baltic has a seasonal variation in abundance both on reference substrates (PVC panels) and on *G. vermiculophylla* thalli. Furthermore, the chemical AF defence against *S. constricta* and *C. tenuicorne* seemingly involves multiple compounds with different polarities and its strength fluctuates seasonally. *Gracilaria vermiculophylla* possibly adjusts its defence against *C. tenuicorne* to the *in situ* presence of *C. tenuicorne*, but not the defence against *S. constricta* to the overall presence of diatoms.

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3. General Discussion

The overall intension of the presented study was to test whether native and non-native populations of *Gracilaria vermiculophylla* differ in their susceptibility to fouling.

I observed that fouling pressure on *G. vermiculophylla* was higher in the non-native compared to the native range (Paper II). Further, I demonstrated that non-native *G. vermiculophylla* were generally better defended against fouling than native conspecifics by both laboratory and field evidences. Specifically, laboratory experiments showed that both living thalli and surface extracts from non-native *G. vermiculophylla* were better defended against both tested groups of fouling organisms, i.e. diatoms and *Ceramium* filaments, regardless of their origin, than those from their native conspecifics (Paper I). Similarly, field experiments showed that at native and non-native study sites, non-native individuals of *G. vermiculophylla* were more resistant to natural fouling than native conspecifics (Paper II). Additionally, the fouling resistance of *G. vermiculophylla* surface extracts against diatoms and *Ceramium* filaments varied with season (Paper I and III) and I could show that the seasonal fluctuations in fouling resistance against *Ceramium tenuicorne* mediated by dichloromethane (DCM)-based polar surface extracts from *G. vermiculophylla* correlated with fluctuations in the intensity of epiphytism by *C tenuicorne* on *G. vermiculophylla* (Paper III). Finally, diatoms were generally deterred by non-polar *G. vermiculophylla* surface extracts made with hexane, while *Ceramium* filaments were generally deterred by surface extracts gained with both hexane and DCM (Paper I and III).

Study questions (see section 1.7) and conclusions:

1. What species can be found in the natural fouling consortia that establish on *G. vermiculophylla* in its native and its non-native range? Do these assemblages generally differ in diversity and biomass?

Florideophyceae (*Ceramium* and *Polysiphonia*) and Ciliata were most common in *Gracilaria*'s native range. Ciliata and Gymnolaemata were most common in its non-native range.

Assemblages in the non-native range were more diverse and abundant. (Paper II)

2. Do native and non-native populations of *G. vermiculophylla* differ with respect to their susceptibility to fouling?

Non-native populations were chemically more defended. (Paper I and II)

3. Does the strength of antifouling defences in *G. vermiculophylla* vary with season and do seasonal fluctuations in the strength of antifouling defences correlate with fouling pressure?

Yes. (Paper I and III)

No, but the strength of the defence against *Ceramium tenuicorne* tended to correlate with fouling pressure exerted by this species. (Paper III)

3.1 Non-native *G. vermiculophylla* are less susceptible to fouling

My study showed for the first time that non-native individuals of *G. vermiculophylla* are better defended against fouling than conspecifics stemming from the native range. This was tested in laboratory and in field fouling experiments (Paper I and II). In general, a difference in fouling rates could either be due to the fact that non-native *G. vermiculophylla* individuals are better defended or due to the fact that non-native macroalgae are less colonized by native foulers. However, since the experiments were conducted in both the native and the non-native range of this alga and both studies showed the same trend, the latter reason could be excluded. My results therefore suggest that non-native *G. vermiculophylla* are better defended against fouling than their native conspecifics.

Interactions between introduced species and their enemies in the new habitat have been considered as an important component determining invasion success (Blossey and Nötzold 1995, Keane and Crawley 2002). However, most studies on non-native species-resident enemy interactions in marine systems have considered only herbivory. In several previous studies, herbivores from the non-native range were found to prefer native over non-native seaweeds (Cacabelos et al. 2010, Enge et al. 2012, Engelen et al. 2011, Weinberger et al. 2008). A low feeding pressure in the non-native range, which potentially mediates a competitive advantage for the non-native species, could explain the later invasion success of introduced seaweeds. However, all previous studies mentioned above were conducted solely within the non-native range of the introduced seaweeds. This was the case until Hierro et al. (2005) argued that experiments that compare the performance of non-indigenous species in their native and in their non-native range are important for testing hypotheses that seek to elucidate factors that determine invasion success. After that, studies on non-native species-enemy interactions seeking to test invasion hypotheses started to use a biogeographical approach. So far, most biogeographical studies on non-native species-enemy interactions again considered herbivory, for which an increased resistance in non-native over native species have been suggested. For instance, it has been confirmed that the non-native brown seaweed *Fucus evanescens* in Sweden (non-native range) was more chemically resistant to herbivory than native conspecifics in Iceland (native range) (Forslund et al. 2010, Wikström et al. 2006). It also has been corroborated that non-native populations of *G. vermiculophylla* had a higher capacity to defend against herbivory than native populations. This was the case in both study areas: Germany (non-native range) and China (native range) (Hammann et al.

2013). Similar to herbivory, epibiosis may also determine invasion success in introduced seaweeds. However, epibiont-basibiont interactions have been widely ignored in invasion ecology and almost all the interactions between non-native basibionts and resident epibionts have only been considered in the non-native range (Baer and Stengel 2014, Strong et al. 2009). So far, defence capacities against fouling between native and non-native populations of the same marine organism have only been compared by Saha et al. (2016) who worked on epibacterial foulers. They suggested that non-native *G. vermiculophylla* are better defended against bacterial epibionts from the non-native range but, at the same time, had reduced their capacity to ward-off epibionts from their home range. My results are in direct contradiction to this finding, what may be due to the use of different microfouler types. Diatoms were employed in my study, while bacteria were used in their case. It is known that bacteria are the first colonizers of bare substrata in the marine environment (Wahl 1989) and bacterial biofilm formation can regulate the further colonization by eukaryote micro- and macrofoulers. The functional differences between epibacteria and other foulers could have led to the evolution of different defence strategies against them in seaweeds. In addition, presumably both, physical and chemical defences, were relevant for the warding-off of foulers in my study, while Saha et al. only identified chemical defence as relevant for their findings.

To my knowledge, mine is the first study to compare the susceptibility to eukaryote micro-epiphytes, macro-epiphytes and natural fouling between native and non-native populations of an aquatic organism. Furthermore, it gives the first example of the scenario that non-native individuals of an aquatic organism are better defended against fouling organisms than native conspecifics and suggests that an enhanced defence against fouling organisms after introduction is a possible reason for the invasion success of *G. vermiculophylla*.

3.2 What makes non-native *G. vermiculophylla* more resistant to fouling?

During the past five to six decades, research on invasion ecology intended to elucidate general mechanisms that explain invasion success in introduced species (Blossey and Nötzold 1995, Callaway and Ridenour 2004, Elton 1958, Keane and Crawley 2002). First studies focused on terrestrial plants and were later followed by studies on marine organisms.

To date, although many studies in invasion ecology dealt with seaweeds, our general understanding of seaweed invasions is still poor. Here, according to my results, I will discuss possible reasons for the enhanced resistant to fouling in non-native over native *G. vermiculophylla* via two approaches: an enhanced antifouling defence capacity after introduction and the possible causes for the enhanced antifouling defence capacity.

3.2.1 An enhanced antifouling defence capacity after introduction

In macroalgae, antifouling defences can be mediated by physical traits, e.g. the thallus surface structure (Chapman et al. 2014, Schumacher et al. 2007), chemical traits. e.g. surface-bound secondary metabolites that have antifouling activities (Nylund et al. 2007, Saha et al. 2011, Thabard et al. 2011), and surface associated bacterial communities that repel foulers (Boyd et al. 1999, Dobretsov et al. 2006).

In the field monitorings in this study, fouling pressure at both native and non-native study sites was generally higher on PVC panels than on *G. vermiculophylla* individuals and only few groups of foulers (e.g. Ciliates) were more abundant on the alga (Paper II). This clearly suggests the presence of a physical, chemical and/or bacterial defence against most of the occurring foulers in *G. vermiculophylla*.

Previous studies showed that surface-associated metabolites of seaweeds can mediate chemical defence against settlement of fouling organisms (Nylund et al. 2007, Rickert et al. 2015, 2016, Saha et al. 2011, 2012) and a chemical anti-macrofouling defence has also been reported for *Gracilaria chilensis*, a species closely related to *G. vermiculophylla* (Lion et al. 2006). However, previous studies did not consider surface-metabolite-based basibiont-epibiont interactions as relevant for our understanding of invasion success in seaweeds (but see Saha et al. (2016)). In my study, both laboratory and field fouling experiments, showed that surface-associated metabolites from non-native *G. vermiculophylla* populations had a higher antifouling capacity compared to those from native conspecifics (Paper I and II). This suggests that an increased level of chemical antifouling defence contributes to the lower susceptibility to fouling in non-native *G. vermiculophylla*. This would support the Novel Weapons hypothesis (Callaway and Ridenour 2004), which suggests that non-native species possess new or stronger defences against resident enemies.

The new technique of enclosing *G. vermiculophylla* in dialysis membrane tubes in field fouling experiments (Paper II) not only hinder *Gracilaria* spores or alga-associated microorganisms from entering the water column, but also excludes any influence of physical antifouling defence properties of *G. vermiculophylla*. This approach thus permits to evaluate the relevance of chemical antifouling defence properties by means of investigating the effect of metabolites released by *G. vermiculophylla* on fouling organisms.

Interestingly, in the laboratory experiments, when comparing the fouling rates of two types of foulers - diatoms as a type of micro-foulers and *Ceramium* filaments as a type of macro-foulers - on living thalli to those on surface extracts coated surfaces, the effect size, i.e. differences in fouling rates, was consistently smaller in surface extracts (Paper I). This confirms that resistance to fouling in *G. vermiculophylla* has, at least partly, a chemical basis. The smaller effect size in the surface extracts could be due to the fact that active metabolites were insufficiently captured by the extraction process or degraded after extraction. Furthermore, it is also possible that some relevant compounds were missed, since only two solvents were used for the extractions (i.e. hexane and DCM). They just cover a limited part of the polarity spectrum. In addition, the compounds which are responsible for the antifouling defences in living *G. vermiculophylla* may not only stem from the algal surface but could also come from the inside of algal cells. This is not unlikely, since some epibionts, including *Ceramium* sp., penetrate into the host and therefore also get in contact with its interior (Leonardi et al. 2006, Martin et al. 2013, Michetti et al. 2016). Finally, other non-chemical traits - which were, of course, excluded in the assays with extracts - could also have contributed to the overall deterrence. Unfortunately, there is no data to elucidate which of these four scenarios was true. However, since seaweeds possess efficient physical and chemical defences to minimize colonization on their body surfaces (see section 1.4, da Gama et al. 2014), it is possible that the increased antifouling activity I observed in non-native *G. vermiculophylla* has either an exclusive chemical basis, which was insufficiently captured by extraction, or was mediated by a multiple (physical and chemical) defence system.

In the laboratory experiments, diatoms from Rongcheng (*Gracilaria*'s native range) generally attached in higher numbers to *G. vermiculophylla* thalli from both distributional ranges than diatoms from Kiel (non-native range), while the opposite was observed when the surface extracts were tested (Paper I). Additionally, in field fouling experiments, the fouling communities that established on dialysis tubes containing *G. vermiculophylla* were more similar to the fouling communities on PVC panels than to fouling communities that developed directly on the surface of this alga (Paper II). All these results indicate that besides a chemical defence, relevant physical antifouling defence mechanisms could exist in

G. vermiculophylla. However, I cannot further tell whether the physical antifouling defence in non-native *G. vermiculophylla* is more active than in their native conspecifics. Physical antifouling defence strategies, such as epithallus sloughing and gelatinous or microstructured surfaces, have been reported from many different macroalgae species (da Gama et al. 2014, Nylund and Pavia 2005, Yamamoto et al. 2013), but have so far not been observed in *G. vermiculophylla* and I also did not investigate this aspect any further.

In my study the observed antifouling capacity cannot be exclusively ascribed to *G. vermiculophylla*. This is because the applied ‘dipping technique’ (de Nys et al. 1998) as well as the technique of enclosing the algal thalli into dialysis membrane tubes do not exclude the influence of surface-associated metabolites from surface-associated microfoulers, such as bacteria and diatoms. Previous studies showed that structural and chemical cues of epibacterial films, such as their microtopography and a range of bacterial products influence fouling on algal surfaces (Qian et al. 2007). Biofilms, for instance, can have inhibitory, inductive or neutral effects on invertebrate larvae attachment (Dobretsov and Qian 2006, Ganesan et al. 2010, Wieczorek et al. 1995). To investigate the role of surface-associated biofilms on the antifouling capacity in *G. vermiculophylla*, I conducted an additional test. In this the attachment rates of *Ceramium* filaments on different densities (0, 1 and 5-fold of the natural density) of surface-associated microfoulers (collected from the surfaces of *G. vermiculophylla* thalli from the Baltic Sea) were quantified. Here the inductive effect increased with the density of surface-associated microfoulers (data not shown) suggesting that the biofilms on Baltic Sea *Gracilaria* populations have a promoting effect on *Ceramium* attachment. Although, I did not assess *Ceramium* attachment rates on biofilms from other *Gracilaria* populations, this finding reveals that the surface-associated microfoulers also influence the colonization of *G. vermiculophylla* by epibionts. I thereby cannot conclude that the antifouling activity observed in surface-associated metabolites from *G. vermiculophylla* thalli is exclusively attributed to *G. vermiculophylla* itself. However, with regard to possible implications of my findings for the invasion ecology of *G. vermiculophylla*, it makes sense to apply the holobiont concept here that views a host alga and its associated microflora as a functional entity (Egan et al. 2013).

3.2.2 Possible causes for the enhanced antifouling defence capacity

Many previous studies have demonstrated that various traits of macroalgae, which mediate resistance against abiotic (such as, desiccation, light and salinity extremes) and biotic (such as, grazers) stressors, also promote their invasion success (Johnson and Chapman 2007). In my study, higher antifouling defence levels were found in non-native than in native *G. vermiculophylla* populations (Paper I and II) and this indicates that the alga's resistance against enemies can change after introduction. Such a change in the defence capacity after introduction could be caused by a directional selection of genotypes that exhibit a higher resistance to fouling. This could happen if the fouling pressure on the translocated organism during transport or after arrival in the new environment is high. When monitoring the natural fouling communities on *G. vermiculophylla* at both native and non-native sites of the alga's distributional range, a lower fouling pressure (fouling colonization on PVC panels) was observed in Rongcheng, China (native range) than in Kiel, Germany (non-native range) (Paper II). I cannot tell whether the fouling pressures at the other sites in the native and the non-native range, at which specimens of *G. vermiculophylla* were collected for this study, followed the same pattern as those in Rongcheng and Kiel. However, in its non-native distributional range *G. vermiculophylla* is mostly found in extremely sheltered lagoons and estuaries (Weinberger et al. 2008), while populations in the native range are often located on wave exposed rocky shores (S. Wang, pers. obs.). It has been reported that wave exposure can generally reduce colonization rates on macroalgae (Koehl et al. 2013) and these environments should therefore be places with a low fouling pressure. The selection of genotypes with a high antifouling defence capacity could have facilitated the settlement and spread of *G. vermiculophylla* in non-native environments with particularly high fouling pressures.

It is also possible that the intense fouling pressure in the non-native habitat induced an increased antifouling defence in *G. vermiculophylla.* Such a demand-driven antifouling defence regulation has been suggested by Saha and Wahl (2013) and Rickert et al (2016). Saha and Wahl (2013) found that the anti-settlement activity of *Fucus vesiculosus* against bacteria in the Western Baltic varied temporally, reaching a peak in summer/autumn, which was in phase with the density of *in situ* bacteria. Rickert et al (2016) showed that in individuals of the two *Fucus* species *F. vesiculosus* and *F. serratus* in the Western Baltic Sea, the chemical defence strengths against microfouling varied seasonally and the fluctuations tend to match with the seasonality in microfouling pressure. In addition, such regulation also was found in my study: a higher fouling pressure exerted by *C. tenuicorne* tended to modulate an increasing antifouling defence in the host *G. vermiculophylla* that reaches its maximum after one month (Paper III). All these evidence therefore increases the possibility that a higher fouling pressure in the non-native habitat could induce an increased antifouling defence in *G. vermiculophylla.*

Furthermore, a change in the strength of antifouling defences could go back to increased energy resources, which are a consequence of the release from other abiotic and biotic pressures in the new environment (Blossey and Nötzold 1995). Under such conditions, non-native seaweeds may reduce specific defences they developed against other pressures in their native range, such as grazing, and shift energy resources towards antifouling defences. The field monitorings hint at the potential relevance of this mechanism: In Rongcheng, China, the amphipod species *Caprella* sp. is the main grazer of *G. vermiculophylla* in many habitats and it can consume substantial parts of the local stock of this alga during summer (S. Wang, pers. obs.). Similarly, in Akkeshi, Japan, local populations of *G. vermiculophylla* are heavily grazed by the amphipod species *Caprella scaura* and *Ampithoe lacertosa* during summer (S. Wang, pers. obs.). However, in Kiel, Germany, so far no herbivore makes use of this alga to such an extent and it seems that the grazing pressure on it is generally lower than in Rongcheng and Akkeshi. However, I do not have information whether the pattern is the same in the other sampled habitats in which we sampled *Gracilaria* in Europe and in Asia. If yes, this could have allowed the non-native *G. vermiculophylla* to reduce the energy budget for anti-herbivory defence and allocate a larger part to antifouling defence. Since it has been reported that non-native *G. vermiculophylla* populations are more defended against herbivores than native populations (Hammann et al. 2013), this scenario is not very likely.

In addition, the increased antifouling capacity in non-native *G. vermiculophylla* could result from an adaptation to other biotic pressures, such as grazing. Non-native *G. vermiculophylla* populations are more defended against herbivores than native populations (Hammann et al. 2013) and compounds that deter consumers are sometimes also active against epibionts. For example, in *Asparagopsis armata*, Bromoform was shown to deter not only mesograzers but also microsettlers (Paul et al. 2006a, Paul et al. 2006b). Likewise, the defence of *G. vermiculophylla* against herbivores is at least partially due to its capacity to produce oxylipins from arachidonic acid after wounding (Hammann et al. 2016) and those compounds were shown to also deter algal epibionts of Gracilarioids (Lion et al. 2006). Non-native populations of *G. vermiculophylla* were already shown to have a higher capacity for producing oxylipins than native populations (Hammann et al. 2016). It has been suggested that oxylipin production requires activation by wounding (Rempt et al. 2012). Since certain foulers of Gracilarioids (ie *Ceramium* species) can cause tissue wounding when they anchor themselves in the host (Leonardi et al. 2006), compounds including oxylipins could also be responsible for the deterrent effects observed in this study.

Finally, since the non-native gene pool is a reduced subset of the gene pool in the donor region of an introduced species (Kim et al. 2010), it is possible that by chance a fouling resistant genotype was highly frequent among the introduced individuals.

In any case, my findings are not in line with the predictions of the ERH and EICA (Blossey and Nötzold 1995, Keane and Crawley 2002), which both suggest that introduced species should experience a reduction in the diversity and the abundance of co-evolved enemies in their new compared to their native range. My field monitorings revealed that the fouling pressure was much higher in Kiel Fjord (non-native range of *G. vermiculophylla*) than in Ailian Bay, Rongcheng (native range), and in the field fouling experiments, higher fouling rates were observed in Kiel Fjord than in Akkeshi Bay, Japan (native range) (Paper II). Interestingly, both investigations provided similar results, although the two study sites in *Gracilara*'s native range were located in different ecozones (Spalding et al. 2007). This suggests that *G. vermiculophylla* faces a more severe fouling pressure in its new environment in the Kiel Fjord. As a matter of fact, most marine fouling organisms are rather unspecific in their host choice (Wahl and Mark 1999) and therefore the probability of being released from specialized fouling organisms after introduction into a new habitat should be low for aquatic organisms. Additionally, the ERH and the EICA do not offer a plausible explanation why local enemies of the new habitat cannot recognize newly introduced species as a resource.

3.3 What cause seasonal variability in the resistance to fouling in macroalgae?

As described in section 1.5, macroalgal antifouling defences can vary seasonally when they are in phase with fluctuations in ambient abiotic (e.g. temperature and light) and biotic (e.g. grazing and fouling pressure) variables (Amade and Lemée 1998, Steinberg and Vanaltena 1992). In my study, I showed that fouling resistance of surface extracts from Baltic Sea *G. vermiculophylla* towards diatoms and *Ceramium* species varied with season (Paper I and III). So far, the related compounds, which are responsible for these observed activities, remain to be determined. The antifouling activities of DCM surface extracts were weakly but significantly correlated with the fouling pressure (determined as *Ceramium* settlement rates on PVC panels) that prevailed shortly before the samples were taken (Paper III). As also descripted in section 3.2.2, this result is somehow in accordance with previous studies, which confirmed that macroalgal antifouling activities exhibit seasonal variation and that such activities can be modulated by fouling pressure (Hellio et al. 2004, Maréchal et al. 2004, Rickert et al. 2015, 2016, Wahl et al. 2010). In addition to this, the listed studies also documented that seasonal variation in macroalgal antifouling activities can be determined by abiotic factors such as water temperature and light intensity. An explanation for this observation could be that increasing irradiance and rising water temperatures lead to an increase in reproduction (mainly in spring) and growth (mainly in summer) of present fouling organisms (Pansch et al. 2012, Wahl et al. 2010). This could, in turn, drive the antifouling capacities of the affected seaweeds (da Gama et al. 2014). Additionally, if the production of antifouling compounds competes with other energy-demanding metabolic functions such as reproduction and growth for the limited resources, increasing irradiance and water temperature could help seaweeds to fuel the production of antifouling compounds (Dworjanyn et al. 2006a, Wahl et al. 2010).

The impact of fouling pressure on antifouling defence strength of host macroalgae may be also reflected by stress-induced shifts of epibiont-basibiont interactions. However, I found no correlational evidence that the mean level of surface colonization by *C. tenuicorne* on *G. vermiculophylla* affected the subsequent mean level of antifouling defences of *G. vermiculophylla* towards the same fouler (Paper III). It is possibly because various other factors that were not controlled may also influence epibiosis, since benthic marine environments are characterized by immense and omnipresent competitions for light, space, nutrients and other resources (Wahl 2009). Macroalgae are rich in organic material and provide safe substrata for colonization, reproduction and habitation. Therefore, the fouling organisms that are present on the limited substratum, will probably interact not only with *G. vermiculophylla*, but also with competing foulers and grazers, which may weaken the correlation between their presence and algal defences. Nonetheless, I found correlational evidence that the recorded antifouling activities of DCM surface extracts from *G. vermiculophylla* against *C. tenuicorne* affected the subsequent settlement rates of *C. tenuicorne* on *G. vermiculophylla*. This is explainable since the increased antifouling defences in algae will reduce the surface colonization of fouling organisms (Dworjanyn et al. 2006b, da Gama et al. 2014).

Interestingly, no correlation emerged in case of resistance toward diatoms. It is possible that this was due to the fact that a single diatom species, i.e. *S. constricta* was used in the bioassays, while *in situ* microfouling pressure goes back to a large group of diatom species. It is also possible that other deterrents in *G. vermiculophylla* that were not extracted, e.g. water-soluble compounds, contributed to the antifouling activities against this species.

3.4 Multiple antifouling strategies in macroalgal metabolites

In terms of production of bioactive natural compounds, macroalgae have provided a large number of original metabolites with a wide range of biological and ecological properties (Puglisi et al. 2014). It has been investigated that macroalgae can produce various metabolites that are broadly defended against a variety of fouling organisms (Schmitt et al. 1995, Othmani et al. 2016).

In my study, I observed that non-polar compounds (extracted with hexane) from *G. vermiculophylla* surfaces had a deterring activity against *S. constricta*. In contrast, both hexane and DCM-based surface extracts inhibited *C. tenuicorne* attachment (Paper I and III). Apparently the deterrent activities of compounds associated with *G. vermiculophylla* surfaces are species-or group-specific, i.e. the compounds which mediate defences against *S. constricta* and *C. tenuicorne* are different and have different polarities. The finding is consistent with previous studies. For example, surface compounds extracted with a mixture of hexane and DCM from *Caulerpa filiformis* significantly inhibited settlement of *Polysiphonia* sp. spores, while DCM-extracted polar compounds inhibited settlement and germling development of *Ulva australis* gametes (Nylund et al. 2007). Further, among several compounds isolated from the Mediterranean brown seaweed *Taonia atomaria*, a glycerol derivative, sn-3-*O*-(geranylgeranyl) glycerol had a significant anti-adhesion effect on bacteria, while a sesquiterpenoid, (-)-gleenol significantly inhibited the adhesion of barnacle cyprids (Othmani et al. 2016). This also becomes apparent when seasonal patterns in the activities of these extracts were compared: the antifouling activity against *S. constricta* of hexane extracts peaked between July and August, while the activity against *C. tenuicorne* peaked between June and July. This suggests that the two organisms were affected by different compounds within the same type of extract. Furthermore, the activity of DCM extracts towards *C. tenuicorne* did not peak between June and July, but between July and August, suggesting that *G. vermiculophylla* employs at least two different compounds of different polarities that both target this epiphyte. Similar results were also showed by Saha et al. (2011, 2012), who demonstrated that surface-associated polar and non-polar metabolites from *F. vesiculosus*, i.e. dimethylsulphopropionate (DMSP), proline and fucoxanthin, have an ecologically relevant role as surface inhibitors against attachments of the same bacterial strains. Furthermore, Othmani et al. (2014, 2016) also showed that both a glycerol derivative, sn-3-*O*-(geranylgeranyl) glycerol and a germacrane, germacra-4(15),5,10(14)-trien-9-ol isolated from the Mediterranean seaweeds had significant anti-adhesion effects on the same bacterial strain.

It is possible that different compounds act respectively and/or synergistically toward the targeted fouling organisms. Regarding *G. vermiculophylla*, at least *C. tenuicorne* seems to be deterred by multiple compounds. However, the surface extracts were obtained with only two solvents (i.e. hexane and DCM) that cover a limited part of the polarity spectrum. It is therefore very likely that there are more deterrents in *G. vermiculophylla* that were not extracted, but which also contribute to the antifouling activities of living individuals. Nonetheless, these results suggest that a number of compounds could, alone or in combination, play a critical role in controlling the fouling on macroalgal surface.

3.5 Conclusion

The conclusions of this thesis comprise three main facets:

First, it is showed for the first time that non-native individuals of a marine organism are better defended against fouling than native conspecifics. Then, it is confirmed that at least a part of the antifouling defence in *G. vermiculophylla* is based on surface associated metabolites. In general, my findings do not support the ERH and EICA, since a higher fouling pressure was observed at a location in the non-native range than in the native range of *G. vermiculophylla*. However, they could support the Novel Weapons hypothesis, because a stronger antifouling defence was observed in non-native *G. vermiculophylla.*

Second, the antifouling defences in *G. vermiculophylla* varied seasonally. I found some hints for the assumption that *G. vermiculophylla* can adjust its antifouling defenses to fouling pressure.

Third, the chemical antifouling defence in *G. vermiculophylla* involves multiple compounds with different polarities.

The existence of multiple defence strategies against fouling in marine organisms allows a better understanding of basibiont-epibiont interactions and the responses of organisms to fluctuating environmental factors. My study gives the first example of a comparison of the susceptibility to eukaryote microfouling, macrofouling and natural fouling between native and non-native populations of an aquatic species and provides new insights into understanding invasion success by basibiont-epibiont interactions in invasion ecology.

3.6 Questions and outlook for the future

There are some questions that still need to be answered and I do not believe we have the answers today:

1. Does *G. vermiculophylla* possess a physical antifouling defence?

2. What is the origin of active polar and non-polar metabolites - *G. vermiculophylla* itself, surface associated biofilms, or both?

3. Are the fouling resistance mediated by *G. vermiculophylla* itself and/or biofilms stronger on non-native or native *G. vermiculophylla*?

4. What are the active metabolites served as the fouling deterrents in native and non-native *G. vermiculophylla* and/or their biofilms? And what are the finally active metabolite(s) responsible for the enhanced antifouling activities in non-native *G. vermiculophylla*?

With time, more and more marine species will be introduced to habitats from which they were previously absent and will spread there, with potential negative impacts on ecosystems and economical values worldwide. A better understanding of the foundations of invasion success in introduced species is important to develop strategies for managing biological invasions. In addition to this, from an ecological perspective, similar as the relationships between corals and associated microbes, the resulting assemblage of the host macroalgae and associated microbes should be regarded as a functional entity or unit, also termed as ‘holobiont’ which has been increasingly used in the past decade (Barott et al. 2011, Egan et al. 2013). Microfouling may profoundly change the mechanical, physical and chemical properties of the body surface of macroalgae and, consequently, all interactions potentially linked in some way to these properties. Numerous case studies with respect to macroalgae-bacteria interactions have demonstrated that associated microbes are an important functional interface to the physiological and ecological performance of the macroalgal host (Egan et al. 2013, Hollants et al. 2012, Singh and Reddy 2014). Therefore, there is an increasing scientific interest to understand the influence of macroalga-associated microbes on micro- and macro-fouling.

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5. Erklärung

Hiermit erkläre ich, dass ich die vorgelegte Dissertation mit dem Titel „Is fouling a determinant of invasion success? - A comparison of the susceptibility to fouling between native and non-native populations of *Gracilaria vermiculophylla*" selbständig und ohne unerlaubte Hilfe angefertigt habe. Ich habe die Arbeit noch keinem anderen Fachbereich bzw. noch keiner anderen Fakultät vorgelegt und habe die Dissertation nach den Regeln guter wissenschaftlicher Praxis verfasst.

Kiel, den